Application of natural deep eutectic solvents in isolation of anthocyanins and biocatalysis with lipase

Panić, Manuela

University of Zagreb

FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

Manuela Panić

APPLICATION OF NATURAL DEEP EUTECTIC SOLVENTS IN ISOLATION OF ANTHOCYANINS AND BIOCATALYSIS WITH LIPASE

DOCTORAL DISSERTATION

Zagreb, 2020

University of Zagreb

FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

Manuela Panić

APPLICATION OF NATURAL DEEP EUTECTIC SOLVENTS IN ISOLATION OF ANTHOCYANINS AND BIOCATALYSIS WITH LIPASE

DOCTORAL DISSERTATION

Supervisor:

PhD Ivana Radojčić Redovniković, Full Professor

Zagreb, 2020

Sveučilište u Zagrebu

Prehrambeno-biotehnološki fakultet

Manuela Panić

PRIMJENA PRIRODNIH EUTEKTIČKIH OTAPALA U IZOLACIJI ANTOCIJANA I BIOKATALIZI POMOĆU LIPAZE

DOKTORSKI RAD

Mentor:

prof.dr.sc. Ivana Radojčić Redovniković

Zagreb, 2020.

This doctoral thesis was made in the Laboratory for Cell Technology, Application and Biotransformations, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb under the mentorships of PhD Ivana Radojčić Redovniković, Full Professor, as a part of scientific research project "Green solvents for green technologies" (GREEN; no.9550; HRZZ) led by Ivana Radojčić Redovniković, PhD, Full Professor.

Ivana Radojčić Redovniković
Pierottijeva 6, 10000 Zagreb
Tel.: + 385 1 46 05 015
email: irredovnikovic@pbf.hr
Scientific personal identification number: 266812

Ivana Radojčić Redovniković was born on 1st of October in Beograd. After degree in Biotechnology at Faculty of Food Technology and Biotechnology, University of Zagreb, she obtained her PhD (topic: Localization of aliphatic glucosinolate biosynthesis and their potential role in plant defence) in 2007 at the same Faculty. Currently, she is working as a full professor in a Laboratory for Cell Technology, Application and Biotransformations at the Faculty of Food Technology and Biotechnology, University of Zagreb. She is conducting several courses for students at the undergraduate and graduate university study programmes and she is the coordinator of two modules at the postgraduate study. She was a mentor of 32 graduate students and 17 bachelor students. Prof. Ivana Radojčić Redovniković published 66 scientific papers listed in CC and SCI scientific database (1401 citations, h-index 18 according to Web of Science). Furthermore, her 6 papers published in the last five years fall into the group of 1% of the most cited papers in the field according to the ISI Web of Knowledge, which indicate the significant contribution of her scientific research work, especially in the field of application of natural eutectic solvents as new environmentally friendly solvents for the extraction of bioactive compounds from various plant materials and in biocatalysis. She participated on 35 international conferences among which 5 was invited lecture. Prof. Ivana Radojčić Redovniković was leader of several projects including "Green solvents for green and biotechnology" founded by Croatian Science Foundation. Currently, she is project leader of „Rational design of natural deep eutectic solvents for chiral drugs preparation and formulation "fund by Croatian Science Foundation and bilateral projects one with Republic of Austria (Natural deep eutectic solvent for biocatalytic preparation of chiral synthons by using alcohol-dehydrogenases) and with Republic of China (Phenolic compounds extraction from plant-endophytes with green solvents for green technologies). She has reviewed over 60 scientific papers in an international journal and in proceedings. Prof. Ivana Radojčić Redovniković was also reviewer for more than 25 different international scientific projects, including Bio-Based Industries Joint Undertaking Call in 2016 & 2019. She has received several awards: Faculty of Food Technology and Biotechnology Award for Total Scientific Contribution - for Achieving High Quality in Scientific Work 2019, International Prizes for Innovation in Green Extraction of Natural Products for Young Scientists, GENP2018 Awards, Silver Medals for Scientific Achievement for Work "Green waste for green technologies" awarded at the 14th ARCA 2016 International Exhibition of Innovations, New Ideas, Products and Technologies. She is a secretary of the Croatian Society for Biotechnology and a member of the Croatian Society of Food Technologists, Biotechnologists and Nutritionists and the European Biotechnology Thematic Network Association (EBTNA).

*First of all, I would like to express my sincere gratitude to my mentor, Professor **Ivana Radojčić Redovniković**, for the continuous support during my Ph.D. study and related research, for her patience, motivation, and immense knowledge. Her guidance helped throughout the whole time of my research and writing of this thesis. I am grateful for her friendly and easy-to-talk-to approach. I could not have imagined having a better mentor for my Ph.D. study.*

*Besides my mentor, I want to thank Associate Professor **Kristina Radošević,** as she had recognized my hard-working attitude in my student days, recommended me for a Ph.D. in Green solvents for green technology group, and worked with me on biological evaluation of green solvents and extracts.*

*I would also like to thank Assistant Professor **Marina Cvjetko Bubalo,** who worked with me on the application of NADES in biocatalysis. Without her precious support, it would not be possible to conduct this research.*

Those three women have done a great job!

*A huge thanks to Professor **Giancarlo Cravotto**. I am grateful and appreciative of the fact that he always finds the time for me in his always-busy schedule. I am also grateful for educational meetings and for accepting me as one of his own.*

*Apart from my Supervisor, I would like to express my gratitude to the rest of the Laboratory for Cell Technology, Application and Biotransformations team – Professor **Višnja Gaurina Srček**, Associate Profesor **Igor Slivac**, and Ph.D. students **Marijan** and **Mia** – for their insightful comments and encouragement, but also productive discussions which incented me to widen my research from various perspectives. Besides professional help, thank You for being my friends.*

*My sincere thanks also go to Associate Professor **Ana Jurinjak Tušek**, Dr. **Marin Roje**, and Dr. **Maja Majerić Elenkov**, who always answered my questions and gave me the advice and comments on my research.*

*A big thanks to all members of the **University of Turin** group, who really helped me to fit in, taught me a lot of things and engaged me in their work. In addition, my thanks go to all members of the **Department for Biochemical Engineering, University of Zagreb** for all their help and kindness.*

I would like to thank the Committee members and the Governance of the Faculty of Food Technology and Biotechnology, which have enabled me to defend my Ph.D. thesis regardless of the current epidemiological situation in the Republic of Croatia and worldwide.

*Thanks to my sister **Tatjana** for following me on my Ph.D. journey through her Master's journey. No words are needed; she knows. Thanks to our parents, grandparents, cousins, oncle and aunts for moral support.*

*I must not forget **Lucija**, **Veronika**, **Laura**, **Ines**, **Martina**, **Kruno**, **Jurica, Dario**, and all of my other friends who have always been there for me. Thank You for your friendship!*

*Last but not least, I would like to thank **Igor**, for selfless help and support in private life, for believing in my research, but also for being critical. I should not forget to thank him for help with NADES Design.*

The theme of the doctoral thesis was accepted at the 8th regular meeting of the Faculty Council of the Faculty of Food Technology and Biotechnology, University of Zagreb in the academic year 2017./2018. held on May 22, 2018 in Zagreb, and the Senate of the University of Zagreb adopted a decision approving the initiation of the process of PhD in the doctoral program on 10th July 2018 at the 13th extraordinary session of the Senate, held in 349. academic year (2017./2018.).

BASIC DOCUMENTATION CARD

University of Zagreb **Ph.D. thesis**
Faculty of Food Technology and Biotechnology
Postgraduate University Doctoral Study in Biotechnology and Bioprocess Engineering, Food Technology and Nutrition
UDK: 547.262: 547.458.84: 577.15: 602.4: 633.63(043.3)
Scientific Area: Biotechnical Sciences
Scientific Field: Biotechnology

APPLICATION OF NATURAL DEEP EUTECTIC SOLVENTS IN ISOLATION OF ANTHOCYANINS AND BIOCATALYSIS WITH LIPASE

***Manuela Panić*, M.Sc.Eng.**

Thesis performed at Laboratory for Cell Technology, Application and Biotransformations, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb
Supervisor: PhD. Ivana Radojčić Redovniković, Full Professor

Short abstract: In this dissertation, 15 natural deep eutectic solvents were successfully prepared and applied for developing an eco-friendly method for extraction of anthocyanins and lipase-catalyzed (*R*)-1-phenylethanol synthesis. Also, the biological activity of prepared grape pomace extracts was evaluated. With natural deep eutectic solvents efficiency of the process was increased in comparison with conventional processes. Extraction and biocatalytic process were optimised using the response surface methodology afterward laboratory-developed methods were scaled up. In both processes, the recyclation of used natural deep eutectic solvents was performed, as well as isolation and purification of the final product (anthocyanins, (*R*)-1-phenylethanol).

Number of pages: 113
Number of figures: 16
Number of tables: 23
Number of references: 141
Original in: English

Key words: (*R*)-1-phenylethanol; biological activitiy; grape pomace; green chemistry; green extraction; natural deep eutectic solvents; Novozyme 435; polyphenolic compounds; solvent recycling; stereoselective biocatalysis

Date of the thesis defense: 8th April, 2020

Reviewers:
1. PhD. Marina Cvjetko Bubalo, Assistant professor
2. PhD. Giancarlo Cravotto, Full Professor
3. PhD. Karin Kovačević Ganić, Full Professor
4. PhD. Marin Roje, Senior scientific associate (substitute)

Thesis deposited in: Library of Faculty of Food Technology and Biotechnology, Kačićeva 23. and National and University Library, Hrvatske bratske zajednice bb. and University of Zagreb, Trg maršala Tita 14.

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu **Disertacija**
Prehrambeno-biotehnološki fakultet
Poslijediplomski sveučilišni studij Biotehnologija i bioprocesno inženjerstvo, prehrambena tehnologija i nutricionizam
UDK: 547.262: 547.458.84: 577.15: 602.4: 633.63(043.3)
Znanstveno područje: Biotehničke znanosti
Znanstveno polje: Biotehnologija

PRIMJENA PRIRODNIH EUTEKTIČKIH OTAPALA U IZOLACIJI ANTOCIJANA I BIOKATALIZI POMOĆU LIPAZE

***Manuela Panić*, mag.ing.bioproc.**

Rad je izrađen u Laboratoriju za tehnologiju i primjenu stanica i biotransformacije na Prehrambeno-biotehnološkom fakultetu Sveučilišta u Zagrebu
Mentor: prof.dr.sc. Ivana Radojčić Redovniković

Kratki sažetak disertacije: U sklopu doktorske disertacije uspješno je pripremljeno i okarakterizirano 15 prirodnih eutektičkih otapala i primijenjeno za razvoj ekološki prihvatljivih metoda ekstrakcije antocijana iz komine grožđa te biokatalitičkog procesa priprave (*R*)-1-feniletanola pomoću lipaze. Također, evaluirana je biološka aktivnost pripremljenih polifenolnih ekstrakata u prirodnim eutektičkim otapalima. Primjenom prirodnih eutektičkih otapala u ekstrakciji polifenolnih spojeva i biokatalizi ostvarena je veća učinkovitost procesa u odnosu na konvencionalne postupke. Ekstrakcija i biokataliza su optimirani primjenom metode odzivnih površina te su laboratorijski razvijene metoda prenijete u veće mjerilo. U oba procesa razvijena je i metoda reciklacije korištenog prirodnog eutektičkog otapala, kao i izolacije i pročišćavanja produkta (antocijani, (*R*)-1-feniletanol).

Broj stranica: 113
Broj slika: 16
Broj tablica: 23
Broj literaturnih navoda: 141
Broj priloga: 1
Jezik izvornika: engleski

Ključne riječi: (*R*)-1-feniletanol; biološka aktivnost; komina grožđa; Novozyme 435; polifenolni spojevi; prirodna eutektička otapala; reciklacija otapala; stereoselektivna biokataliza; zelena ekstracija; zelena kemija

Datum obrane: 8. travnja 2020.

Stručno povjerenstvo za obranu:

1. doc.dr.sc. Marina Cvjetko Bubalo
2. prof.dr.sc. Giancarlo Cravotto
3. prof.dr.sc. Karin Kovačević Ganić
4. dr.sc. Marin Roje, viši znanstveni suradnik (zamjena)

Rad je pohranjen u knjižnici Prehrambeno-biotehnološkog fakulteta u Zagrebu, Kačićeva 23. i u Nacionalnoj i sveučilišnoj knjižnici u Zagrebu, Hrvatske bratske zajednice bb. i Sveučilištu u Zagrebu, Trg maršala Tita 14.

ABSTRACT

Many industrial processes use large quantities of volatile, flammable and toxic organic solvents based on unsustainable resources such as oil, resulting in significant environmental and economic impacts. Over the past decade, natural deep eutectic solvents (NADES) have become promising alternatives to traditional organic solvents from both environmental and technological perspectives. The properties that have gained them the environmentally friendly label are nonvolatility (reduced air pollution), nonflammability (process safety) and excellent stability (potential for recycling and reuse). The number of structural combinations encompassed by NADES is tremendous, thus it is possible to design NADES with unique physicochemical properties for a particular purpose such as the design of solvents for efficient extraction of biologically active compounds or biocatalytic pocess. It is interesting to find the natural deep eutectic solvents structures that will enable highly efficient processes. The aim of this dissertation is to prepare several natural deep eutectic solvents based on renewable sources and to apply these solvents in industrial interesting processes: (1) isolation of anthocyanins from grape pomace; (2) lipase-catalysed (*R*)-1-phenylethanol synthesis. Further, since NADES showed great potential for application in lab scale of above mentioned processes, and there are no commercial NADES-based process in existence, final goal of this dissertation was to formulate the postulates for the development of eco-friendly technology with natural deep eutectic solvents and their implementation in industrial processes. Furthermore, besides mentioned advantages of NADES application, according to economic demands, used solvent in industrial scale should be possible to recycle. Therefore, in both processes' recyclation and reuse methodology for used NADES was developed. Prepared polyphenolic extracts in NADES was also considered as ready-to-use in industry without downstream purification steps.

Keywords:

(*R*)-1-phenylethanol; biological activitiy; grape pomace; green chemistry; green extraction; natural deep eutectic solvents; Novozyme 435; polyphenolic compounds; solvent recycling; stereoselective biocatalysis

PROŠIRENI SAŽETAK

PRIMJENA PRIRODNIH EUTEKTIČKIH OTAPALA U IZOLACIJI ANTOCIJANA I BIOKATALIZI POMOĆU LIPAZE

U novije vrijeme velika pozornost pridaje se zelenim i održivim tehnologijama te njihovoj primjeni u različitim granama industrije. Stoga je akademska zajednica potaknuta razvijati nove, sigurnije i energetski učinkovitije procese proizvodnje i primjene kemikalija koji se zasnivaju na prihvatljivom kompromisu između ekonomskih, socijalnih i ekoloških zahtjeva, opisanih kao zelena kemije. U okviru zelene kemije od velike je važnosti razvoj i pronalazak odgovarajuće zamjene za sveprisutna štetna organska otapala koja se koriste u različitim industrijama. Zbog svojstva poput nehlapljivosti i nezapaljivosti, posljednjih se godina intenzivno izučavaju prirodna eutektička otapala (eng. Natural deep eutectic solvents, NADES) kao moguća nova zelena otapala. Prirodna eutektička otapala su smjese kvaternih amonijevih soli (npr. kolin-klorid) s donorom vodika (npr. alkoholi, organske kiseline, šećeri, vitamini i amidi) u definiranom stehiometrijskom odnosu povezanih vodikovim vezama. Karakteristične su po svojstvu eutektičnosti – tj. sniženo im je talište u odnosu na tališta zasebnih ishodnih metabolita. Pored zelenog karaktera, jedna od istaknutih karakteristika ovih otapala jest mogućnost modifikacije njihove strukture, a time i promjena fizikalno-kemijskih svojstava što posljedično utječe na mogućnost i učinkovitost njihove primjene. Budući da je broj mogućih kemijskih struktura ovih otapala iznimno velik, mogućnost njihova dizajniranja za specifične namjene čini ih vrlo zanimljivim za uporabu u različitim granama industrije te se istražuju moguće primjene ovih otapala u sintetskoj kemiji, elektrokemiji, izradi nanomaterijala, biokemiji, biokatalizi te izolaciji i analizi različitih spojeva što uključuje i biološki aktivne spojeve iz biljaka.

Jedna od zanimljivih primjena prirodnih eutektičkih otapala je ekstrakcija biološki aktivnih spojeva, kao što su polifenoli. Polifenolni spojevi privukli su pažnju znanstvenika zbog pozitivnih učinaka na ljudsko zdravlje i postoji veliki interes za njihovu upotrebu u farmaceutskoj, kozmetičkoj i prehrambenoj industriji. Stoga je posljednjih nekoliko godina ekstrakcija i identifikacija polifenolnih spojeva iz različitih prirodnih izvora postala vrlo važno područje znanstvenih istraživanja. Zbog složene strukture ne postoji jedinstvena metoda za ekstrakciju svih biljnih polifenolnih spojeva, a konvencionalni postupci ekstrakcije obično su dugotrajni te se povezuju s velikom potrošnjom organskih otapala i nedovoljnim iskorištenjem ekstrakata. S obzirom da eutektička otapala imaju mogućnost otapanja i polarnih i nepolarnih komponenti, mogu se koristiti za vrlo učinkovitu ekstrakciju spojeva iz biljaka uključujući i

polifenolne spojeve. Nadalje, NADES-ovi se sastoje od jednostavnih, jeftinih i prirodno dostupnih spojeva s prihvatljivim profilom toksičnosti koji su prisutni u našoj svakodnevnoj prehrani, zbog čega se pretpostavlja da bi se pripremljeni ekstrakti mogli izravno koristiti u prehrambenoj, farmaceutskoj, kozmetičkoj i agrokemijskoj industriji bez dodatnih pročišćavanja.

Nadalje, prirodna eutektička otapala mogu se primijeniti i u kemo- i regio-stereoselektivnim biotransformacijama, koje se razvijaju u standardnu zelenu tehnologiju zbog zahtjeva za visokim stupnjem enantiomerne čistoće biološki aktivnih kiralnih spojeva ili kiralnih sintona u proizvodnji lijekova te poljoprivrednih i ostalih kemikalija. Naime, interakcija kiralnih spojeva s kiralnim okruženjem u biološkim sustavima može rezultirati biološki različitim, ponekad i suprotnim učincima enantiomera te je ovakva čistoća proizvoda od izuzetne važnosti. Jedna od glavnih prednosti primjene prirodnih eutektičkih otapala je dizajniranje idealnog otapala za biokatalitički sustav te dobra kontrola/poboljšanje topljivosti supstrata/produkta, brzine reakcije, aktivnosti, enantioselektivnosti, regioselektivnost i stabilnosti biokatalizatora.

Bez obzira na obećavajuće karakteristike, NADES-ovi još uvijek nisu našli primjenu u industriji. Kako bi se dizajnirala učinkovita metoda primjenom NADES-a, potrebno je uključiti sljedeće korake: (*i*) priprema i karakterizacija NADES-a, (*ii*) odabir NADES-a, (*iii*) optimiranje NADES-a (*iv*) reciklacija NADES-a i (*v*) prijenos laboratorijski razvijenog procesa u veće mjerilo. Unatoč navedenim prednostima primjene prirodnih eutektičkih otapala, vodeći se ekonomskim aspektima, korišteno otapalo u industrijskom mjerilu treba moći reciklirati. Dosad je opisano nekoliko načina izolacije molekule od interesa i reciklacije otapala, kao što su primjena anti-otapala, rekristalizacija i adsorpcijska kromatografija, no, budući da je tlak pare prirodnog eutektičkog otapala gotovo jednak nuli, izolacija krajnjeg proizvoda još uvijek predstavlja problem za industrijsku primjenu.

Zbog gore navedenog ciljevi ovog rada bili su:

1. Pripraviti i okarakterizirati odabrana prirodna eutektička otapala.
2. Razviti ekološki prihvatljivu metodu ekstrakcije antocijana iz komine grožđa primjenom prirodnih eutektičkih otapala i alternativnih izvora energije.
3. Razviti ekološki prihvatljivu metodu za sintezu (*R*)-1-feniletanola pomoću lipaze u prirodnim eutektičkim otapalima.

4. Ispitati mogućnost reciklacije i ponovne primjene prirodnih eutektičkih otapala u navedenim procesima.

METODOLOGIJA RADA

Istraživanja su obuhvatila metodologiju za: (*i*) razvoj ekološki prihvatljivog postupka izolacije antocijana iz komine grožđa primjenom prirodnih eutektičkih otapala te ispitivanje biološke aktivnosti pripremljenih ekstrakata (*ii*) razvoj ekološki prihvatljivog postupka za pripravu (*R*)-1-feniletanola pomoću lipaze u prirodnim eutektičkim otapalima.

Metodologija za razvoj ekološki prihvatljivog postupka izolacije antocijana iz komine grožđa pomoću prirodnih eutektičkih otapala i alternativnih izvora energije

Klasičnim postupcima sinteze (miješanje na magnetskom mješaču s grijanjem) pripravila su se odabrana eutektička otapala iz prirodnih izvora (npr. kolinijeve soli, ugljikohidrati, alkoholi, organske kiseline i aminokiseline) za ekstrakciju antocijana iz komine grožđa.

Pripremljenim eutektičkim otapalima određena su fizikalno-kemijska svojstva (polarnost, viskoznost, pH-vrijednost) te im je ispitan citotoksični učinak na stanicama sisavaca (HeLa, MCF-7 i HEK293T) dobivenim iz banke stanica (ATCC), a proliferacija stanica određena je MTS-metodom. Rezultati su prikazani kao EC_{50} vrijednosti, odnosno koncentracije prirodnog eutektičkog otapala koja izaziva inhibiciju rasta 50 % populacije stanica.

Nakon sinteze, fizikalno-kemijske i biološke karakterizacije eutektičkih otapala, odabrano je najpogodnije prirodno eutektičko otapalo za ekstrakciju antocijana iz komine grožđa te je učinkovitost ekstrakcije uspoređena s konvencionalnim otapalom (zakiseljeni etanol). Identifikacija i kvantifikacija antocijana iz pripremljenih ekstrakata provedena je pomoću tekućinske kromatografije visoke djelotvornosti (HPLC) (Agilent 1200 Series HPLC system (Agilent, San Jose, CA, USA).

Stabilnost antocijana u prirodnim eutektičkim otapalima, koja su se pokazala najboljima s obzirom na učinkovitost ekstrakcije, i konvencionalnim otapalima praćena je na -18 °C, 4 °C i 25 °C inkubacijom kroz 60 dana. Uzorci su izuzimani u određenim vremenskim periodima te su antocijani iz pripremljenih ekstrakata identificirani i kvantificirani pomoću HPLC uređaja.

Prilikom odabira najboljeg otapala u obzir je uzeta učinkovitost ekstrakcije, stabilnost antocijana u pripremljenim otapalima te cijena prirodnih eutektičkih otapala.

Nakon odabira najboljeg otapala, odabrana je i metoda ekstrakcije. Ekstrakcija je optimirana metodom odzivnih površina: sustavno je ispitan utjecaj ključnih parametara (temperatura, vrijeme ekstrakcije, udio vode u otapalu, utjecaj ultrazvučnog i mikrovalnog zračenja) na uspješnost ekstrakcije.

Iz pripremljenih ekstrakata, adsorpcijskom kromatografijom razdvojeni su antocijani i prirodno eutektičko otapalo korišteno za pripremu ekstrakata. Kao nosač korištena je makroporozna smola na koju su se adsorbirali antocijani iz ekstrakta. Zaostalo prirodno eutektičko otapalo sa stacionarne faze je eluirano deioniziranom vodom, a antocijani sa zakiseljenim etanolom. Iz frakcije u kojoj je zaostalo reciklirano otapalo otparen je višak vode te se je regenerirano otapalo ponovno koristilo u ekstrakciji antocijana iz komine grožđa.

Prema optimalnim uvjetima laboratorijski razvijene metode ekstrakcije potpomognute utjecajem ultrazvuka i mikrovalova, ekstrakcija atocijana iz komine grožđa pomoću eutektičkog otapala kolin-klorid:limunska kiselina (ChCit) je provedena u većem mjerilu.

Na kraju, procijenjena je biološka aktivnost ekstrakata komine grožđa ispitivanjem njihove biološke aktivnosti na humanim tumorskim staničnim linijama (HeLa; MCF-7) radi mogućnosti korištenja ekstrakata komine grožđa u ChCit bez izolacije i pročišćavanja polifenola iz otapala. Antiproliferacijski učinci ispitivanih ekstrakata određen je MTS metodom. Pojava apoptoze i zastoj staničnog ciklusa praćem je metodom protočne citometrije (Muse® Cell Analyzer, EMD Milipore Corporation, Massachusetts, USA) uz primjenu Muse™ Annexin V & Dead Cell kita i Muse™ Cell Cycle kita. Pripremljenim ekstraktima određen je i antioksidacijski kapacitet ORAC metodom, a mjerenja su provođena na Varian Cary Eclipse Spectrofluorimetru (Palo Alto, CA, USA) te mogućnost zaštite stanica od oksidativnog stresa metodom protočne citometrije uz primjenu Muse™ Oxidative Stress kita.

Metodologija za razvoj ekološki prihvatljivog postupka priprave (*R*)-1-fenil etanola pomoću lipaze u prirodnim eutektičkim otapalima

Sinteza i karakterizacija prirodnih eutektičkih otapala za sintezu (*R*)-1-feniletanola pomoću lipaze provedena je kao što je opisano u „Metodologija za razvoj ekološki prihvatljive metode izolacije antocijana iz komine grožđa pomoću prirodnih eutektičkih otapala i alternativnih izvora energije“.

Nakon pripreme i karakterizacije prirodnih eutektičkih otapala, odabrano je najpogodnije prirodno eutektičko otapalo za proizvodnju (*R*)-1-feniletanola hidrolizom (*R,S*)-1-feniletil acetata pomoću lipaze (*Candida antarctica* lipaza B imobilizirana na makroporozne

akrilne kuglice – Novozym 435) kao biokatalizatora. Enantioselektivna hidroliza se je provodila šaržno na vrtložnoj miješalici uz prirodna eutektička otapala kao otapalo. Također, uspješnost enantioselektivne hidrolize u zelenim otapalima uspoređeno je s puferom kao konvencionalnim reakcijskim medijem.

Usporedba eutektičkih otapala kao otapala u lipazom kataliziranoj enantioselektivnoj hidrolizi (*R, S*)-1-feniletil acetata temeljena je na utjecaju kemijske strukture prirodnog eutektičkog otapala na aktivnost (prikazano kao inicijalna brzina reakcije) i enantioselektivnost enzima.

Molarna koncentracija nastalog alkohola praćena je pomoću plinskog kromatograma s MS detektorom (GCMS QP2010 PLUS) primjenom kapilarne kiralne kolone Varian (CP-CHIRASIL-DEX CB 25 mx0,25mmx0,25 μm).

U najpovoljnijim otapalima ispitana je i stabilnost Novozym 435 koji je inkubiran kroz 30 dana na sobnoj temperaturi. Rezultati su prikazani kao rezidualna aktivnost lipaze.

Nakon odabira najpovoljnijeg otapala ispitan je i utjecaj ključnih parametara biokatalitičkog procesa poput količine biokatalizatora, omjera supstrat/produkt, temperature i udjela vode u eutektičkom otapalu primjenom metode odzivnih površina.

Ispitana je i mogućnost reciklacije otapala, izolacije i pročišćavanja višekratne uporabe enzima. Nakon provođenja reakcije, Novozym 435 je izdvojen iz reakcijske smjese i ponovno korišten u nekoliko ciklusa. Također, reciklirano otapalo se je ponovno koristilo za hidrolizu (*R,S*)-1-feniletil acetata.

Na kraju, prema optimalnim uvjetima laboratorijski razvijene metode, sinteza (*R*)-1-feniletanola u eutektičkom otapalu kolin-klorid:glicerol (ChGly) je provedena u većem mjerilu prema optimalnim uvjetima iz laboratorijskog mjerila.

REZULTATI I RASPRAVA

S obzirom na dosadašnje spoznaje, cilj ove disertacije bio je primijeniti prirodna eutektička otapala za razvoj ekološki prihvatljivog postupka izolacije antocijana iz komine grožđa te sintezu (*R*)-1-feniletanola pomoću lipaze. Nadalje, unatoč navedenim prednostima primjene prirodnih eutektičkih otapala, vodeći se ekonomskim aspektima, idealno otapalo za industrijsku primjenu treba moći reciklirati i ponovno koristiti, stoga je u oba procesa ispitana i mogućnost reciklacije prirodnih eutektičkih otapala i njihova ponovna upotreba.

U prvom dijelu ovog doktorskog rada prikazani su rezultati vezani uz primjenu prirodnih eutektičkih otapala i alternativnih izvora energije u ekstrakciji antocijana iz komine grožđa, nusproizvoda nastalog proizvodnjom vina. Eksperimentalni dizajn uključivao je sljedeće korake: (*i*) odabir prirodnog eutektičkog otapala, (*ii*) odabir i optimiranje metode ekstrakcije, (*iii*) izolacija i pročišćavanje antocijana iz pripremljenog ekstrakta i (*iv*) prijenos laboratorijski razvijene metode ekstrakcije i izolacije antocijana u veće mjerilo (0,5 L). Najprije, pripremljeno je i okarakterizirano 8 različitih prirodnih eutektičkih otapala (kolin-klorid:limunska kiselina (ChCit), kolin-klorid:jabučna kiselina (ChMa), kolin-klorid:prolin:jabučna kiselina (ChProMa), prolin:jabučna kiselina (ProMa), betain:jabučna kiselina (BMa), betain:limunska kiselina (BCit), jabučna kiselina:glukoza:glicerol (MaGlcGly), jabučna kiselina:glukoza (MaGlc)). Pripremljena otapala su polarna, s polarnošću sličnom vodi, pH-vrijednosti od 0,49 - 3,27. Viskoznost otapala je u rasponu od 0,0169 - 0,0817 Pas. Pripremljena otapala nisu inhibirala rast stanica HEK239T, HeLa i MCF-7 u rasponu od 0 - 2000 mg L^{-1} te se mogu smatrati netoksičnima. Najveći maseni udio antocijana ekstrahiran je s ChCit ≈ ChProMa ≈ EtOH > ChMa ≈ Bcit ≈ MaGlcGly ≈ ProMa ≈ BMa > MaGlc. Udio ekstrahiranih antocijana bio je u rasponu od 0,28 do 0,92 mg $g_{s.tv.}^{-1}$. Kako učinkovitost ekstrakcije nije bila statistički značajno različita (p<0,05) između ChCit, ChProMa i etanola, ispitana je stabilnost antocijana u tih otapalima kroz 60 dana na -18 °C, 4 °C i 25 °C. U prirodnom eutektičkom otapalu ChCit uočena je značajna stabilnost s obzirom na etanol, gdje su se pri temperaturama 4 °C i -18 °C nakon 60 dana antocijani degradili samo za 14 %, s obzirom na etanol gdje su bili degradirani 30 %. S obzirom na karakterizaciju otapala, učinkovitost ekstrakcije i stabilnost antocijana u otapalu, ChCit je odabrana za daljna istraživanja. Nadalje, ispitan je utjecaj alternativnih izvora energije za poboljšanje učinkovitosti ekstrakcije. Osim korištenja zelenih otapala u ekstrakciji, zelena kemija predlaže i korištenje alternativnih izvora energije. Tako se među mnogim metodama ekstrakcije ističe ekstrakcija potpomognuta istovremenim ultrazvučnim i mikrovalnim zračenjem. Ovakav način ekstrakcije osigurava skraćenje procesa, smanjenje utroška energije i povećanje prinosa. Ekstrakcija potpomognuta istovremenim zračenjem mikrovalova i ultrazvuka bila je najučinkovitija (1,1 mg $g_{s.tv.}^{-1}$). Ekstrakcija je i optimirana primjenom metode odzivnih površina te su optimalni uvjeti: snaga mikrovalova 300 W, vrijeme ekstrakcije 10 min i udio vode u ChCit 30%, v/v bili najučinkovitiji u ekstrakciji. Pri tim uvjetima, izolirano je 1,8 mg $g_{s.tv.}^{-1}$. Iz ekstrakta pripremljenog prema optimalnim uvjetima, izolirani su antocijani adsorpcijom na makroporoznu smolu Sepabeads825L, nakon čega su desorbirani s 75 %-tnim zakiseljenim etanolom. Uspješnost izolacije bila je 99,46 %. ChCit je eluiran s kolone s destiliranom vodom

te je višak vode otparen. Uspješnost recilacije ChCit bila je 96,8 %. Prema laboratorijski razvijenoj metodi, ekstrakcija i izolacija antocijana iz komine grožđa uz reciklaciju otapala, provedena je i u većem mjerilu. Pri tom je ekstrahirano 1,58 mg $g_{s.tv.}^{-1}$ antocijana, a uspješnost izolacije antocijana i reciklacije otapala bila je 90 %, tj. 77.91 %. U ovom dijelu istraživanja, razvijena je metoda za ekstrakciju antocijana primjenom NADES-a koja je dobar temeljem za industrijsku primjenu.

Nadalje, pripremljen ekstrakt komine grožđa pomoću 30 % (v/v) eutektičkog otapala ChCit razmatran je i za direktnu primjenu u industriji, bez izolacije i pročišćavnja, te je ispitana biološka aktivnost ekstrakta. Određen je profil polifenola te antioksidacijski kapacitet primjenom ORAC metode. Ispitana je *in vitro* biološka aktivnost na humanim tumorskim staničnim linijama (HeLa i MCF-7). Primjenom analizatora staničnog zdravlja Muse™ i odgovarajućeg kita određen je tip stanične smrti kao i mogućnost ekstrakta za zaštitu stanica od oksidativnog stresa. Svi rezultati su uspoređeni su s ekstraktom pripremljenom pomoću zakiseljenog etanola. Uz pomoć ChCit ekstrahirana je veća količina polifenola, uspoređujući s etanolom (2892,07 ± 60,12 mg $kg_{s.tv.}^{-1}$ za GPChCit tj. 2647,48 ± 53.33 mg $kg_{s.tv.}^{-1}$ za GPEtOH). Antioksidacijski kapacitet pripremljenih ekstrakata bio je 2189,97 ± 15,07 μmol Trolox ekvivalenta g $_{s.tv.}^{-1}$ za ekstrakt pripremljen s ChCit (GPChCt) tj. 1229,65 ± 56 μmol Trolox ekvivalenta g $_{s.tv.}^{-1}$ za ekstrakt pripremljen pomoću etanola (GPEtOH), što je u korelaciji s ukupnim izoliranim polifenolnim spojevima. Nadalje, ispitan je antiproliferativni i antioksidacijski utjecaj pripremljenih ekstrakata. Prilikom tretmana HeLa i MCF-7 stanica ekstraktom GPEtOH, nije uočen inhibitorni učinak na proliferaciju stanica dok je s GPChCit inhibirano ~ 60 % stanica s obzirom na kontrolne, netretirane stanice. S obzirom na uočen antiproliferacijski učinak stanica tretmanom s GPChCit, primjenom analizatora staničnog zdravlja Muse™ i odgovarajućeg kita određen je tip stanične smrti. Rezultati su pokazali da inhibicija rasta HeLa stanica uzrokavana tretmanom GPChCit nije zbog apoptoze, nego zastoja staničnog ciklusa što može uzrokovati nekrozu. Ispitana je i mogućnost ekstrakata komine grožđa za zaštitu stanica od oksidativnog stresa. Uočen je 20 % veći postotak preživljenja stanica tretiranih s GPChCit nakon izazivanja oksidativnog stresa, u usporedbi s kontrolnim netretiranim stanicama ROS (+) stanica je bilo 10 % manje nego u kontrolnim stanicama).

U drugom dijelu istraživanja prirodna eutektička otapala primijenjena su za sintezu važnog kiralnog građevnog bloka (*R*)-1-feniletanola, koji se koristi kao sintetski intermedijer za fine kemikalije u farmaceutskoj i agrokemijskoj industriji. U svrhu rada pripravljeno je sedam različitih prirodnih eutektičkih otapala (kolin-klorid: glukoza (ChGlc), kolin-klorid:

etilen glikol (ChEG), kolin-klorid:glicerol (ChGly), glukoza:glicerol (GlcGly), glukoza:etilen glikol (GlcEG), sorboza:etilen glikol (SorEG), etilen glikol:glukoza:fruktoza (EGGlcFru)). Sva pripremljena otapala bila su nisko toksična (EC_{50}>2000 mg L^{-1}). Priprmljeni NADES-ovi bili su blago kiseli (pH-vrijednost od 3,3 – 6,25). Nadalje, pripremljena i okarakterizirana otapala primijenjena su za *Candida antarctica* lipaza B (Novozyme 435) kataliziranu sintezu (*R*)-1-feniletanola. Iz rezultata, vidljivo je da je enantiomerni višak reakcije u svim ispitanim prirodnim eutektičkim otapalima ~ 99 %, što znači da je korištena lipaza stereospecifična za nastajanje (*R*)-alkohola. Inicijalna brzina rekacije, konverzija i produktivnost bili su u rasponu od 0,1323 - 0,369 mmoL L^{-1} min^{-1}, 11,69 - 41,14% i 0,00456 - 0,01905 mmoL L^{-1} min^{-1}. Kako produktivnost rekacije nije bila statistički različita u ChEG, ChGly, ChGlc i kalij-fosfatnom puferu kao referentnom otapalu ($p < 0.005$), ispitana je i stabilnost enzima u tim otapalima kroz 28 dana. Nije uočena značajno razlika u stabilnosti u ispitanim otapalima, osim u ChGly gdje je rezidualna aktivnost nakon 28 dana bila ~80 % s obzirom na aktivnost bez inkubacije, u usporedbi s puferom gdje je bila ~40 %. S obzirom na rezultate aktivnosti i stabilnosti enzima, kao i na fizikalno-kemijska svojstva otapala , ChGly izabran je kao otapalo za sintezu (*R*)-1-feniletanola. Odabir otapala, već je veliki korak pri optimiranju biokatalitičke metode s obzirom da fizikalno kemijska svojstva utječu na aktivnost enzima, ali su dodatno, primjenom metode odzivnih površina optimirani vrijeme i temperatura reakcije, te udio vode u kolin-klorid:glicerolu. Optimalni uvjeti za maksimalnu postignutu konverziju reakcije 50,14 % bili su 5 h i 52 min, 50,4 °C te 47,75 % (v/v) vode u otapalu ChGly. Nakon laboratorijski optimirane metode, sinteza (*R*)-1-feniletanol u ChGly provedena je u većem mjerilu, gdje je konverzija reakcije bila 54,57 %. Na kraju, izoliran je proizveden (*R*)-1-feniletanol ekstrakcijom tekuće - tekuće s etil - acetatom te pročišćen kolonskom kromatografijom s silikagelom kao stacionarnom fazom, dok je mobilna faza bila eitl-acetat. Iskorištenje pročišćavanje je 81,24 %. ChGly je pritom recikliran uz 90,74 %-tno iskorištenje te je ponovno korišten. Recikliran je i biokatalizator u 5 idućih ciklusa te je konverzija reakcije nakon petog ciklusa bila jednaka kao i sa nerecikliranim enzimom. Postupak reciklacije otapala i enzima te pročišćavanja produkta proveden je i nakon biokatalize u većem mjerilu. U ovom djelu istraživanja razvijena je ekološki prihvatljiva metoda sinteze (*R*)-1-feniletanola koja je dobar temelj za implementaciju prirodnih eutektičkih otapala u pripravi kiralnih građevnih blokova u industrijsko mjerilo.

ZAKLJUČCI

U ovom radu priremljena su i okarakterizirana prirodna eutektička otapala (NADES) i primijenja u ekstrakciji antocijana iz komine grožđa te u lipazom (*Candida antarctica* lipaza B) kataliziranoj sintezi (*R*)-1-feniletanola. Na temelju provedenih istraživanja i dobivenih rezultata može se zaključiti:

1. 15 prirodnih eutektičkih otapala je pripremljeno i okarakterizirano. Struktura otapala utjecala je na njihova fizikalno kemijska svojstva.
2. Osam pripremljenih otapala primijenjeno je u ekstrakciji antocijana iz komine grožđa u svrhu pronalaska najučinkovitijeg otapala. S obzirom na sve kriterije (fizikalno-kemijska svojstva otaplaa, učinkovitost ekstrakcije, stabilnost antocijana), kolin-klorid:limunska kiselina (ChCit) odabrana je kao otapalo za izolaciju antocijana iz komine grožđa.
3. Ispitan je i utjecaj različitih metoda ekstrakcije na učinkovitost te je najučinkovitija ekstrakcija bila potpomognuta istovremenim mikrovalnim i ultrazvučnim zračenjem. Ekstrakcija je i optimirana metodom odzivnih površina te su optimalni uvjeti bili: snaga mikrovalova - 300 W, ultrazvuka - 50 W, vrijeme - 10 min, te 30 % vode u ChCit te je pritom ekstrahirano 1,8 mg $g_{s.tv.}^{-1}$ antocijana .
4. S obzirom na laboratorijski razvijenu metodu ekstrakcije, u većem mjerilu ekstrakcija je provedena u 2 odvojena koraka: ultrazvučni predtretman komine grožđa u ChCit (500 W, 5 min) te ekstracija potpomognuta mikrovalovima (300 W, 10 min) pri čemu je ekstrahirano 1,58 mg $g_{s.tv.}^{-1}$ antocijana.
5. Antocijani iz pripremljenih ekstrakata su uspješno izolirani i pročišćeni primjenom adsorpcijske kromatografije s makroporoznom smolom Sepabeads825L kao stacionarnom fazom je ChCit pritom reciklirana i ponovno korištena.
6. Procjenjujući biološku aktivnost pripremljenog ekstrakata, jači inhibitorni učinak na HeLa i MCF-7 stanične linije uočen je pri tretmanu ekstraktom komine grožđa u ChCit, u usporedbi s ekstraktom pripljemljenim s etanolnom. Mehanizam antiproliferativnog učinka, određen analizatorom staničnog zdravlja je zastoj staničnog ciklusa. Antioksidacijski kapacitet ekstrakta te mogućnost zaštite stanica od oksidativnog stresa tretmanom s ekstraktom komine grožđa u ChCit je također primijećen.
7. Sedam prirodnih eutektičkih otapala je primijenjeno za lipazom kataliziranu sintezu (*R*)-1-feniletanola. S obzirom na fizikalno-kemijska svojstva otapala, te aktivnost i stabilnost biokatalizatora, ChGly je izabran kao otapalo.

8. Optimirana je i biokatalitička metoda primjenom metode odzivnih površina te su optimalni uvjeti za najveću konverziju (*R*)-1-feniletil acetata u (*R*)-1-feniletanol: 5 h 52 min, udio vode u kolin-klorid:glicerolu 47,74 % te temperatura reakcije 50,4 °C.
9. S obzirom na laboratorijski razvijenu biokatalitičku reakciju, sinteza (*R*)-1-feniletanola je provedena u većem mjerilu (0,5 L) prema optimalnim uvjetima te je konverzija reakcije bila slična kao u manjem mjerilu.
10. Proizvedeni (*R*)-1-feniletanol je uspješno izoliran i pročišćen iz reakcijske smjese primjenom ekstrakcije tekuće-tekuće s etil acetatom te je pritom ChGly recikliran. Nadalje, (*R*)-1-feniletanol i zaostali supstrat (*S*)-1feniletil acetat je pročišćen na silikagelu. Biokatalizator je također recikliran i korišten u idućih 4 ciklusa.
11. Tijekom izrade doktorske disertacije postavljeni su postulati za razvoj ekološki prihvatljivih tehnologija primjenom prirodnih eutektičkih otapala i njihov prijenos u veće mjerilo te znanja stečena ovim istraživanjem predstavljaju doprinos razvoju novih, konkurentnih i ekološki prihvatljivih industrijskih procesa.

Ključne riječi: (*R*)-1-feniletanol; biološka aktivnost; komina grožđa; Novozyme 435; polifenoli; prirodna eutektička otapala; reciklacija otapala; stereoselktivna biokataliza; zelena ekstrakcija; zelena kemija

TABLE OF CONTENTS

1. INTRODUCTION

Nowadays, green and sustainable technologies and their application in different industries is of growing interest. Therefore, scientific community is strongly motivated to develop new, safer and sustainable processes in which chemicals based on compromise between economic, social and environmental requirements would be used. Already in 1991, chemist P.T. Anastas addressed the need for harmful solvents reduction through a specific program called green chemistry. Within green chemistry principles, finding and development of a promising alternative to traditional, toxic organic solvents used in chemical and biotechnology industries is of great importance. Due to the properties such as non-volatility and non-flammability, in the past decade, natural deep eutectic solvents (NADES) have attracted attention as potential new green solvent. NADES are mixtures of cheap, natural, non-toxic and readily available components, as choline chloride alcohols, organic acids, sugars, vitamins and amines. NADES the mixtures obtained by the complexation between a hydrogen acceptor such as non-toxic quaternary ammonium salt (e.g. cholinium chloride) and a naturally-derived uncharged hydrogen-bond donor (e.g. amines, sugars, alcohols and carboxylic acids) in a certain molar ratio, bonded with hydrogen bonds between NADES components. Besides green character, one of the distinctive features of these solvents is the possibility to modify their structure in order to adjust their physicochemical properties. Since the number of possible chemical structures of these solvents is vast, the possibility of their design for specific applications makes them very interesting for use in in various industrial fields such as synthetic chemistry, electrochemistry, making nanomaterials, biochemistry, biocatalysis and isolation and analysis of various compounds such as biologically active compounds from plants.

Over the last few years, the use of natural deep eutectic solvents as a possible green alternative to conventional solvents has been steadily growing in research articles. The number of publications about deep eutectic solvents in 2020 was around 700. All published research data to date refer to the lab scale, and, there are no commercial NADES-based process in existence, although NADES show great potential for industrial application thanks to their acceptable costs, the versatility of their physicochemical properties, simple and inexpensive preparation and low cytotoxicity. In order to design efficient method by using NADES for industrial application, further steps should be included: (*i*) preparation and characterization of NADES, (*ii*) NADES selection, (*iii*) optimization of methods, (*iv*) NADES recovery (*v*) scale-up.

Based on aforementioned, in this dissertation physicochemical and biological characterization of selected NADES were conducted, with the aim of applying solvents with the best properties in an environmentally friendly isolation method of anthocyanins from grape pomace and (*R*)-1-phenylthanol synthesis with lipase. Furthermore, biological activity of grape pomace extracts was evaluated to explore potential for ready-to-use industrial application. Besides mentioned advantages of NADES application, according to economic demands, used solvent in industrial scale should be possible to recycle. Therefore, in both processes' possibilities of recyclation and reuse of NADES was tested. Finally, laboratoy developed methods were scaled-up.

2. THEORETICAL BACKGROUND

2.1. Natural deep eutectic solvents (NADES) concept

Green chemistry is the way of creation and application of chemical products and processes that reduce or eliminate the use or production of substances hazardous to human health and the environment (Anastas, 1991). A growing area of research in the development of green technologies is devoted to designing new, more environmentally friendly solvents which use would meet technological and economical demands. Requirements for alternative solvents are a reasonable price, non-toxicity for humans and environment, non-flammability, biodegradability and possibility for regeneration/recovery (Cvjetko Bubalo et al., 2015; Clark and Macquarrie, 2002). Currently, known green solvents are water, agro solvents, ionic liquids, and deep eutectic solvents. Over the past decade, natural deep eutectic solvents have gained enormous attention from the scientific community, and the number of reported articles in the literature has grown exponentially.

The deep eutectic solvents (DES) was first described by Abbott et al. (2003). DES are a mixture of amides with quaternary ammonium salts that had melting points much lower than those of their pure compounds, due to the formation of a hydrogen bonds between a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) at a well-defned stoichiometric proportion. Later, a few research groups suggested the modification of definition (Martins et al., 2019; Paiva et al., 2018). In the review of Martins et al. (2019), DES is defined as a mixture of two or more pure compounds for which the eutectic point temperature is below that of an ideal liquid mixture, presenting significant negative deviations from ideality ($\Delta T2 > 0$). Beside DES, in literature, those mixtures are sometimes named as low-transition-temperature mixtures (LTTMs) (Francisco et al., 2013). Also, there is a subgroup of DES, formed by compounds from natural sources and therefore named natural deep eutectic solvents (NADES), although sometimes in literature both terms (DES and NADES) are used for the same mixtures (Martins et al., 2019; Ruesgas-Ramon et al., 2017).

NADES fully represent the green chemistry principles owning to their specific properties: (*i*) the cost of NADES is comparable or even lower than conventional solvent; (*ii*) sustainable production with 100% atom economy production, (*iii*) chemical and thermal stability, (*iv*) non-volatility and non-flammability, (*v*) low toxicity and biodegradability. Moreover, NADES probably occur in living cells and are involved in the biosynthesis, solubilisation and storage of various poorly water-soluble metabolites and unstable compounds in cells, playing important physiological and chemical roles (Choi et al., 2011; Liu et al., 2018).

As for NADES environmental footprint, these solvents are considered *a priori* to be non-toxic based on the toxicity data for the components that make up the NADES (Cvjetko Bubalo et al., 2015a; Radošević et al., 2015). But, this theory does not take into account the possibility of a synergetic effect of combining the compounds in the NADES, which could have a significant impact on the biological properties of such mixtures, what was later showed by a number of authors who were first to asses ecotoxicity of NADES (Hayyan et al., 2013; Juneidi et al., 2016; Paiva et al., 2014; Radošević et al., 2015). The effect of various NADES have been evaluated for toxicity on different organism/cell lines: bacteria (e.g. *Eschericia coli*, *Staphilococus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonela enteritidis*), mammalian cell lines (e.g. HeLa, MCF-7, CCO, CaOV3, HepG2), fish (*Cyprinus carpio*), aquatic crustaceans (*Artemia salina*) and plants (wheat seeds *T. aestivum*). Generally, depending on structure, NADES possesses low to moderate toxicity. Many authors suggest that the NADES containing organic acids as the HBD (for instance oxalic, citric, malic, or tartaric acids) exhibit greater *in vitro* cytotoxicity than the NADES containing sugars as the HBD (for instance glucose, mannose, fructose, and xylose), probably due to low pH value of solvents containing acids (Paiva et al., 2014; Radošević et al., 2016b). Several research groups investigated biodegradability of various NADES using microorganisms from urban or industrial effluents and all tested NADES were classified as 'readily biodegradable′ with good correlation between toxicity and biodegradability (again acid-based NADES were degraded slower than others) (Radošević et al., 2015; Wen et al., 2015; Zhao et al., 2015).

2.1.1. Preparation of NADES

Structurally, NADES consists of at least two inexpensive, non-toxic and easily available components which are able to self-associate at a certain molar ratio to form a new eutectic phase characterized by a melting point below 100°C (Cvjetko Bubalo et al., 2015c). Thus, NADES are relatively easily prepared by mixing a HBA (usually choline or ethylammonium chloride), with HBD such as polyalcohol (e.g. ethylene glycol and glycerol), sugar (e.g. glucose, saharose, xylose), sugar alcohol (e.g. xylitol, sorbitol), organic acid (e.g. malic acid, lactic acid) or amide (e.g. urea) at moderate temperature (~50°C) (Figure 2.1.). The interactions between the compounds occur through intermolecular hydrogen bonds which in a certain range of compositions present a strong depression of the melting point, consequently resulting in a liquids-state mixture. To be precise, the charge delocalization occurring is hereafter responsible for the decrease in the melting point of the mixture relative to the melting points of the starting materials (Abbott et al., 2003). A classic example is the mixture of choline chloride (m.p. =

302°C) and urea (m.p.= 133°C) in a 1:2 molar ratio resulting in a room-temperature liquid (m.p. =12°C) (Cvjetko Bubalo et al., 2015). A wide range of salts can be used (choline chloride is being the most used one, with even wider range of HBD which are usually readily available components from natural primary metabolites such as amines, sugars, alcohols, sugar alcohols, polyols and organic acids (Cvjetko Bubalo et al., 2018)

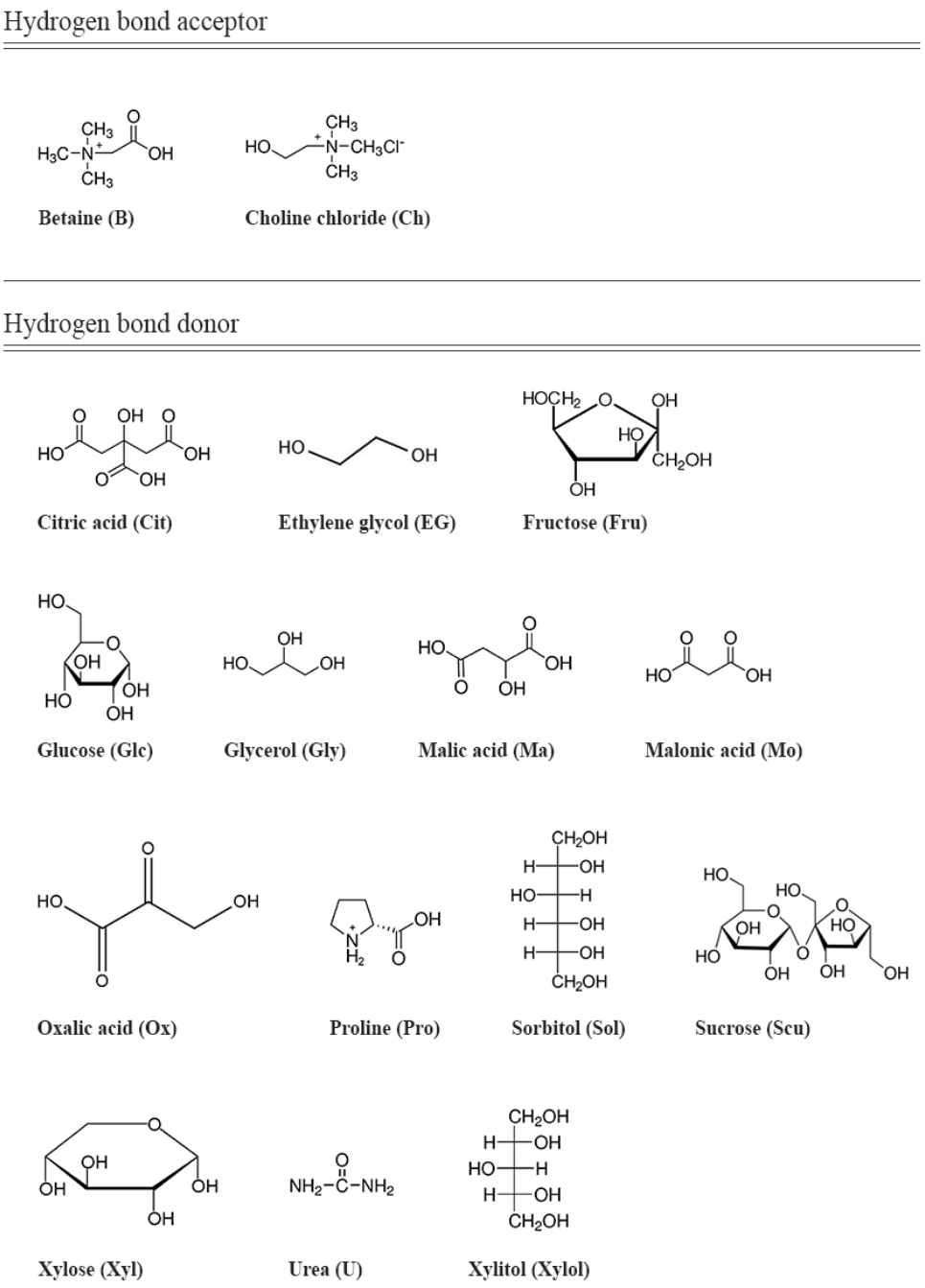


Figure 2.1. Overview of chemical structure of different hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) with the ability to form natural deep eutectic solvents preparation.

The most of the researchers still prepare NADES in their lab prior to further application, thus it is important to mention some basic knowledge regarding preparation procedure and consequent characterization of these solvents.

Regarding the used HBA and HBD (Figure 2.1.), the usual way of labelling the NADES is full name of HBA: full name of HBD. The abbreviations of NADES are formed as first letters of HBA and HBD. For example, for choline chloride:glucose, abbreviation is ChGlc. There is no uniform system for labelling of full and short name of NADES, and in this work, the way described above was used.

The typical preparation proceeds as follows. Firstly, hygroscopic components for NADES preparation needs to be vacuum dried before use (e.g. choline chloride). This is very important as the water in components directly influence their weights and consequently the final molar ratio of components in the mixture. Also, residual water cannot be completely removed ones the NADES is formed since molecules of water also form strong hydrogen bonds with NADES components – making tightly bound molecules of water almost impossible to remove. If the water is essential for further NADES application, water can be added directly with HBD and HBA during preparation, or can be added after the preparation. According to literature, there are few methods of NADES preparation: (*i*) grinding (*ii*) heating and stirring method (usually 50-80°C) for several hours, (*iii*) evaporating method, (*iv*) freeze-drying method, (*v*) using alternative energy sources – microwave and ultrasound (Cvjetko Bubalo et al., 2015c; Florindo et al., 2014; Ruesgas-Ramón et al., 2017). First step in all mentioned methods above is mixing HBA and HBD (optional also water) in certain molar ration. Afterwards, some mechanical, thermal or nonthermal energy source is applied for several hours until liquid is formed. Florindo et al. (2014) reported formulation of by-products during organic-acid based NADES preparation by heating and suggest that the first method of NADES preparation should be grinding at room temperature if it is possible, to avoid degradation of NADES. If the grinding is not working, then mixing at lowest possible temperature is an alternative. Cvjetko Bubalo et al. (2016a) performed microwave- and ultrasound-assisted NADES preparation and reduced preparation time for more than 7-fold compared to conventional heating method. Some authors perform the preparation in the inert atmosphere using nitrogen (Gutiérrez et al., 2011). After the preparation it is recommended to confirm the structure and purity of NADES with ^{1}H-HMR, DSC, and FT-IR analysis (Cvjetko Bubalo et al., 2015a; Mitar et al., 2019; Paiva et al., 2018; Panić et al., 2019a). The prepared NADES should be properly sealed until further application to prevent their interaction with air. It is recommended to store the NADES in closed vials or

exicator because the prepared NADES can also bond water from atmosphere. Majority of authors prepare commonly used NADES reported in the literature, but for preparation of novel NADES, extensive search for adequate components and the molar ratio between them to form eutectic mixture at room temperature (or up to 50 °C) is required (Mitar et al., 2019).

Herein at the end of this section it should be mentioned that though it is sometimes relatively easy to find an appropriate NADES for certain process, this is occasionally ungrateful procedure. Namely, sometimes is difficult to predict which mixtures and in which molar ratios will originate as a NADES and also which solvent properties crucial will be exerted. Therefore, the composition of NADES and their physical, thermal, chemical or biological properties should be characterized on case by case scenario. Abundance of possible NADES formulations, especially when possible addition of certain amount of water is taken into account, makes it impossible to prepare and characterize all of them, pointing out the need for development of predictive NADES structure-activity mathematical models. Approaches used so far for screening appropriate/ideal NADES (out of enormous pool of structural possibilities) have been governed by time consuming empirical methods, while systematic investigation in NADES structure-activity relationship, as a backbone for rational design of these solvents, is still lacking.

Current literature suggest COSMO-RS, software developed by Klamt and co-workers that can predict thermodynamic properties and phase equilibrium which would help in designing the best possible solvent for a certain application (Eckert and Klamt, 2002; Palomar, 2010). COSMO-RS can design new solvent structures with tailored properties and thus the trial and error method of NADES preparation can be avoided (Abranches et al., 2019).

2.1.2. Physicochemical characteristics of NADES

NADES are consider highly viscous and dense at room temperature, which negatively impact handling or mixing in processes involving them (Mitar et al., 2019). In general, sugar-based NADES with choline chloride as HBA are the most viscous and dense, while polyalcohol-based are less. For example, at room temperature, density and viscosity of choline chloride:glucose (1:1) with 30% of water is 1.176g cm^{-3} and 0.0226 Pas and of choline chloride:ethylene glycol (1:2) with 30% of water is 1.092 g cm^{-3} and 0.0069 Pas (Mitar et al., 2019, 2018). Additionally, polarity of solvent is important for application of solvents in wanted industrial process. It is well established that choline chloride- based NADES containing organic acid as HBD are the most polar, while sugar and polyalcohol-based NADES are the least polar. For example, polarity of choline chloride:citric acid with 30% of water is 49.39 kcal mol^{-1}, of

choline chloride:ethylene glycol 50.37 kcal mol^{-1} and chloride:glucose 50.63 kcal mol^{-1} (Panić et al., 2019a). All mentioned characteristics could be adjusted by addition of water (discussed in more detail afterwards). For example, the addition of water in NADES leads to a decrease in the viscosity and density of NADES (Cvjetko Bubalo et al., 2016b; Mitar et al., 2019). However, it should be noted that a large excess of water in the NADES could break the halide-HBD supramolecular complex and a simple aqueous solution of the individual components could be obtained by losing H-bond network (Faggian et al., 2016; Mitar et al., 2019). The high viscosity of NADES can be a restrictive factor for using NADES as extraction solvents on industrial scale not only because of lower extraction efficiencies, but also due to the energy required for stirring and pumping. Beside polarity, viscosity and density, important property of solvent for further application is pH value. As expected, the nature of HBA and HBD determines the acidity and basicity of the NADES. Organic acid-based NADES are by their nature acidic (pH<3), whereas other mentioned NADES belong to the group of weakly acidic to neutral solvents with pH>4 or basic with pH >7 (Paiva et al., 2014). Also, increased pH values with increasing water content were visible in eutectic mixtures with extremely low pH-values, while pH values of eutectic mixtures with pH in the upper region of acidity decreased with increasing water content (Mitar et al., 2019).

NADES show great potential for industrial application thanks to their acceptable costs, the versatility of their physicochemical properties, simple and inexpensive preparation and low cytotoxicity. Only Abbott et al. (2007) have reported a scaled-up process that was performed using NADES ChGly for biodiesel purification. When the scale-up possibilities are deliberated, many factors need to be discussed and taken into consideration, including both the economic and environmental issues of NADES, as well as the exaction methods (Bosiljkov et al., 2017). The production of NADES can be classified as sustainable processes, but also price of the solvents should be bear in mind, when scale-up is one of final process goal. In general, economical evaluation of the DES showed that the average price is around 0.85 – 11.05 \$ kg^{-1}, comparable to organic solvent. Nevertheless, a great difference in the NADES price was noticed (Bogaars, 2015). According to Panić et al. (2017) who calculated NADES price according to the website of Merck (Germany), NADES containing polyalcohols are twice more expansive than the one containing amino acids. For example, among NADES investigated the cheapest solvent was choline chloride:ethylene glycol at molar ratio 1:2 (43.72€ kg^{-1}) while the most expensive one was choline chloride:proline:malic acid at molar ratio 5:2 (252.42 € kg^{-1}). At the same time, it is well known that NADES have almost zero vapour pressure, which minimises the risks of air pollution by NADES, in contrast to organic solvents. Then again, this property

of NADES might be a huge problem when the recovery of the target compounds is needed, particularly on an industrial scale (paragraph 2.2.3).

2.2. Application of NADES in development an eco-friendly extraction method

Nowadays, a number of articles on NADES and their application on extraction of biological active compounds as flavonoid, phenolic acid, anthocyanins, terpenoids, alkaloids and saponins are available (Cvjetko Bubalo et al., 2018). Beside green solvents in extraction, green extraction suggest discovery and design of extraction processes which will reduce energy consumption, allows use of renewable natural products, and ensure a safe and high-quality extract/product described in 6 principles. Three major solutions have been identified to design and demonstrate green extraction on laboratory and industrial scale to approach an optimal consumption of raw materials, solvents and energy: (*1*) improving and optimisation of existing processes; (*2*) using non-dedicated equipment; and (*3*) innovation in processes and procedures but also in discovering alternative solvents. Beside use of alternative solvents (principle 2), green extraction principles are: innovation by selection of varieties and use of renewable plant resources (principle 1), reduce energy consumption by energy recovery and using innovative technologies (principle 3), production of co-products instead of waste to include the bio- and agro-refining industry (principle 4), reduce unit operations and favour safe, robust and controlled processes (principle 5) and aim for a non-denatured and biodegradable extract without contaminants (principles 6) (Chemat et al., 2012).

In order to design efficient extraction method by using NADES, following steps should be included: (*i*) selection of NADES physicochemical characteristic, (*ii*) selection and optimization of extraction method and, (*iii*) recovery of target compounds form NADES if it is necessary (*iv*) scale-up of extraction method.

2.2.1. Selection of NADES physicochemical characteristics

NADES capacity to extract biological active compounds varies considerably due to physiochemical properties of each NADES. Selection of NADES cannot be based only on extraction efficiency because many other properties may also get involved in final decision such as pH, viscosity, price, molar ratio of NADES forming compounds, environmental impact, etc. (Ruesgas-Ramón et al., 2019). To select a suitable extraction solvent, different types of NADES containing organic acid, polyalcohol or sugar as HBD, which considerably differ in physicochemical properties, should be tested. First step is selection of NADES component which could be later modified by changing molar ratio between component and/or by addition

of water (Bakirtzi et al., 2016; Bosiljkov et al., 2017; Chanioti and Tzia, 2018; Dai et al., 2013a; El Kantar et al., 2019; Lakka et al., 2019; Ruesgas-Ramón et al., 2019). For example, Ruesgas-Ramón et al. (2019), screened six different NADES (choline chloride or betaine-based one with glycerol, ethylene glycol and lactic acid as hydrogen bond donor) for the extraction of target molecule from coffee, cocoa by- and co-products and showed important effect of the NADES composition on extraction efficiency, with the choline chloride:lactic acid being the most promising one. The extraction efficiency in this article was correlated with polarity of NADES. This corresponds to general principle "like dissolves like," i.e., that that polar compounds are better extracted by polar solvent and vice versa (Ruesgas-Ramon et al., 2017).

Beside HBD selection, different molar ratio in NADES can also improve extraction efficiency (Bakirtzi et al., 2016; Cvjetko Bubalo et al., 2018; Jeong et al., 2015b). Zhang et al. (2014) reported that molar ratio of choline chloride:glycerol from 1:2 to 1:4 increased significantly the recovery of catechin from green tea. Probably, this could be due to decreased in the amount of choline chloride which led to reduction of proportion of hydrogen bond receptors in the NADES system negatively influencing major mechanism of solubility of bioactive compounds. Namely, NADES have ability of donating and accepting protons and electrons which confers them the ability to form hydrogen bonds, thus increasing their dissolution capability (Bubalo et al., 2018; Ruesgas-Ramon et al., 2017).

Further improvement of extraction efficiency could be obtained by optimization of NADES water content (Ruesgas-Ramon et al., 2017). Addition of water leads to decrease in the viscosity of NADES, thus enhancing the mass transfer from plant matrices to solution and increasing extraction efficiency. Viscosity of NADES in generally very high at room temperature which is one of the major problems when using NADES as extraction solvents, so that some NADES cannot be even used in extraction without addition of water due to problems of NADES transferring and filtering (Cvjetko Bubalo et al., 2018; Cvjetko Bubalo et al., 2016b). On the other hand, addition of water causes a decrease in hydrogen bonding interactions between NADES and target components. Moreover, large excess of water in NADES could break the halide-HBD supramolecular complex and a simple aqueous solution of the individual components could be obtained (Dai et al., 2015). In general, NADES with high water content are better for polar compounds and NADES with low water content are more suitable for the extraction of less polar compounds (Dai et al., 2013a).

Furthermore, stability of target compounds in NADES should be considered as important factor for NADES selection while NADES is better medium for stability of polyphenols (Cvjetko Bubalo et al., 2018; Dai et al., 2014). Such conclusion was reported by Dai et al.

(2016) who studied stability of anthocyanins in *Catharanthus roseus*- or grape pomace- extracts prepared with NADES and conventional solvents (ethanol and water). Authors implied higher stability for anthocyanins in lactic acid:glucose than in conventional solvents. Stabilisation ability of NADES correlated with strong hydrogen bonding between solutes and solvent molecules while among tested NADES sugar-based ones showed the best results. This interaction decreases the movement of solute molecules, reducing its contact time with oxygen and the interface of NADES and air, and consequently reducing oxidative degradation, the major degradation mechanism (Bubalo et al., 2018; Dai et al., 2016).

2.2.2. Biological evaluation of NADES-based extracts

The positive biological aspects of polyphenols, such as the fruit's antioxidant, antimicrobial, anti-inflammatory and anticancer properties, as well as the cardiovascular protection the fruit is well known (Teixeira et al., 2014; Xia et al., 2010). Moreover, if biological activity of plant extract is one of the research interest possible biological activity of NADES should be considered. Previous studies indicate that NADES could enhance the antioxidative activities of plant extracts, which could be explained by the reactive oxygen species scavenging activities of the NADES itself or NADES forming compounds (Nam et al., 2015; Radošević et al., 2015). The antioxidative activity of these NADES was not unexpected because some forming compounds (e.g. malic acid, citric acid, proline and betaine) also possess antioxidative activity (Tang et al., 2013). It has been proposed that NADES formed from compounds with proven pharmacological effects, such amino acid or organic acid, could also have similar properties indicating that not only physicochemical characteristics of solvents, but also their biological activity, could be fine-tuned (Radošević et al., 2016c). Another interesting feature of NADES is its potential biological activity since it was proposed that is possible to design NADES with specific biologically activity (Dai et al. 2015). For example, if you need solvent with antioxidative or/and antitumor activity you could simply prepare NADES with compounds that possess desired biologically active. Previously, it was demonstrated that NADES used for extraction purposes could enhance the antioxidative activities of obtained plant extracts, which could be explained by the reactive oxygen species scavenging activities of the NADES itself or NADES forming compounds (Nam, et al., 2015; Radošević et al., 2016a). Furthermore, since the components of NADES are abundant in the nature and are part of our daily diet, as well as a food supplements already present on the market (e.g. choline, citic acid, betaine, amino acids, etc.) it is expected that extract obtained by NADES may be directly used in food,

pharmaceutical, cosmetical and agrochemical products without the need for expensive downstream purification steps (Dai et al. 2013).

2.2.3. Selection and optimization of extraction method

NADES choice is followed by selection of extraction method which also significantly contributes to the extraction efficiency. When methods for green extraction are considered reduction of energy consumption by using innovative technologies (e.g. ultrasound extraction (UAE), microwave extraction (MAE), negative pressure cavitation method) should be taken into account. These innovative technologies have been recognized as excellent energy source promoting extraction efficiency (Chemat et al., 2019).

UAE enhancement of extraction yield could be explained with ultrasound propagation in a solid/liquid media. Cavitation phenomena leads to high shear forces in the media. The implosion of cavitation bubbles on a product's surface results in micro-jetting which generates several effects such as surface peeling, erosion and particle breakdown. Additionally, implosion of cavitation bubbles in a liquid media leads to macro-turbulences and to a micro mixing (Chemat et al., 2017). Also, shockwave-induced damage of plant cell wall occur causing releasing cell content into the solvent (Cravotto and Cintas, 2007). On the other hand, ultrasonic waves could cause some changes in chemical composition as a consequence of target compound degradation and production of free radicals within the gas bubbles (Cravotto and Cintas, 2007).

In MAE, the process acceleration and high extraction yield may be the result of a synergistic combination of two transport phenomena: heat and mass gradients working in the same direction. On the other hand, in conventional extractions the mass transfer occurs from inside to the outside, although the heat transfer occurs from the outside to the inside of the substrate. In addition, although in conventional extraction the heat is transferred from the heating medium to the interior of the sample, in MAE the heat is dissipated volumetrically inside the irradiated medium (Chemat and Cravotto, 2012).

Several studies showed that NADES are compatible with those technologies and optimization of process parameters by experimental design based on response surface methodology were often conducted (Table 2.1.). Irrespectively of extraction methods chosen, process parameter optimized were commonly exaction time, extraction temperature, solvent to solid ratio, particle size of matrix water content in NADES (Bakirtzi et al., 2016; Bosiljkov et al., 2017; Cvjetko Bubalo et al., 2016b).

It is well known from the literature data that not only the extraction conditions, but also the characteristics of the samples can affect the efficiency of the extraction, either independently

or interactively. Particle size is one of the important factors that influence the extraction process. The extraction yield is higher for smaller particles, due to shorter diffusion path length and enhancement of the surface area with particle size reduction. If the particles are too small, however, they can pose problems of channelling inside the extraction bed, which can cause efficiency and yield losses (Bosiljkov et al., 2017).

The extraction temperature is often a critical parameter as polyphenols are very unstable and are susceptible to degradation at higher temperatures. Cvjetko Bubalo et al. (2016) tested the extraction efficiency of anthocyanins from grape skin with NADES in dependence on extraction temperature and showed that the extraction efficiency of anthocyanins significantly increased when the temperature was raised from 50 to 65 °C, whereas further temperature increase caused decrease in anthocyanin contents.

Usually NADES containing different water contents is optimised in order to reduce viscosity. In many cases addition of water between 20-30% (w/w) beneficially influences extraction yield of both polar and non-polar compounds (El Kantar et al., 2019). However, NADES viscosity could be modified with process temperature, since it is known that viscosity is correlated with temperature. Higher temperature leading to more decrease in viscosity, and thus enhancing the dissolution of the target molecules (Dai et al., 2013a).

Table 2.1. Some recent studies of phenols extracted using NADES.

Screened NADES	Method of extraction	Biomass	Target compund (the most promising solvent)	References
ChCit;ChOx; ChMa;ChGlc; ChFru; ChXyl; ChGly	UAE Optimal conditions: 30.6 min, 341.5 W, 35.4% water (v/v)]	Wine lees	Total anthocyanin (ChMa, 20% of water)	(Bosiljkov et al., 2017)
LaCh; LaNaOAc; $LaNH_4Oac$; LaGln; LaGln	UAE Optimal conditions: 140W, 80 ◦C, 90 min, 20 % water (v/v)]	Greek medicinal plants – dittany (D), fennel (F), marjoram (M), mint (MN) and sage (S)	Total flavonoids: D (LaGln) F ($LaNH_4OAc$) M ($LaNH_4OAc$) MN (LaCh) S (LaNaOAc)	(Bakirtzi et al., 2016)
ChCi; ChMa; ChGly; FruGlc; MaGlc; BScu; BCit; BMaGlc; CitFruGlc; MaGlcFru; LMa; SerMa; AlaCit; LaP; LaFru	Mechanical stirring Optimal conditions: 25% of water in NADES	Vanilla pods	Vanillin (LaFru, LaP)	(González et al., 2018)
ChGly; ChGly; ChOx; ChMa; ChSor; ChProMa	Investigated method: microwave-assisted extraction (MAE); ultrasound-assisted extraction methods (UAE)[a] Optimal conditions: 65°C, 50 min, 25% water (w/w)	Grape skin	Quercetin-3-O-glucoside (ChGly, 50% of water) Malvidin-3-O-mono glucoside (ChOa, 25% of water) Malvidin-3-O-acetylmonoglucosides (ChOx, 25% of water) Malvidin-3-(6-O-*p*-coumaroyl) monoglucosides (ChOx, 25% of water) Peonidin-3-(6-O- *p* coumaroyl)monoglucosides (ChOx, 25% of water) Petunidin-3-O-monoglucoside(ChOx, 25% of water) Peonidin-3-O-monoglucoside(ChOx, 25% of water) Cyanidin-3-O-monoglucoside (ChProMa, 50% of water)	(Cvjetko Bubalo et al., 2016b)

			Delphinidin-3-O-monglucoside (ChProMa, 25% of water) (+)-Catechin (ChOx, 25% of water)	
ChLa; BLa; ChGly; BGly; ChB1,4; BB1,4	Investigated method: heat stirring-assisted extraction (HSE)[a] or ultrasound-assisted extraction (UAE) Optimal conditions: 3min, US power = 200W	Coffee pulp (CP), cocoa husk (CH), and pod husk (CPH)	Total polyphenolic content (CP- ChLa; CH-ChLa; CPH-EtOH)	(Ruesgas-Ramón et al., 2019)
ChCit; ChLa; ChMalt; ChGly	Mechanical stirring [a], microwave – (MAE), ultrasound – (UAE), high hydrostatic pressure –assisted extractions(HHPAE) Optimal conditions: 20 % v/v of water in NADES	Olive pomace	Total phenolic content (ChCit and ChLa)	(Chanioti and Tzia, 2018)
ChMa; MaGlc; ChGlc; MaPro; GlcFruScu; GlyProScu	Mechanical stirring+UAE Optimal conditions: 40°C; 1h; 20%, v/v of water in NADES	*Ginkgo biloba leaves, (GB)* and *Panax ginseng (PG)*	Phenolics, terpenoids and phenolic acids GB (ChMa and GlyProScu) PG (ChMa and MaGlc)	(X. Liu et al., 2018)

[a] the best extraction method

Component abbreviations: NH_4OAc, ammonium acetate; Ala, β-alanine; B, betaine; B1,4, 1,4-butanediol; Ch, choline chloride; Cit, citric acid; Fru, fructose; Glc, glucose; Gln, glycine; Gly, glycerol; L, L-Serine; La, lactic acid; Ma, malic acid; Malt, maltose; Ox, oxalic acid; P, 1,2-propanediol; Pro, proline; NaOAc,sodium acetate; Ser, L-serine; Sor, sorbose; Suc, sucrose; W, water; Xyl, xylose.

2.2.4. Recovery of target compounds from NADES

Besides mentioned advantages of NADES application, according to economic demands, used solvent in industrial scale should be possible to recycle. Current literature suggests several possibilities for target-compound recovery and NADES recycling such as addition of antisolvent, recrystallisation, liquid-liquid extraction and adsorption chromatography but since the NADES have very low vapour pressure, isolation of final product is still the biggest problem for further industrial application of those solvents (Jeong et al., 2015a; Panić et al., 2019a; Ruesgas-Ramon et al., 2017).

In a few studies polyphenols were isolated from NADES-extracts with liquid-liquid extraction. The first step in such approach is finding non-miscible solvents with NADES-based extracts. In most cases that are unwanted volatile organic solvents (e.g. *n*-hexane, *n*-heptane, toluene, diethyl ether, etc.), but in more recent papers, also the use of green agro-solvents is mentioned (e.g. ethyl acetate, 2-methyl-tetrahydrofuran, ethyl lactate, etc.). In this method, polyphenols from NADES was extracted to organic solvent. The weaker hydrogen-bonds between polyphenols and NADES are, the better extractions from the NADES. Usually, several steps of adding fresh solvent are required. Organic phases are collected and evaporated, resulting in polyphenols in dry weight. Advantages of this method is easy NADES recycling, but negative side is huge volumes of aprotic solvent use and low solubility of polyphenols in aprotic solvent (Ruesgas-Ramon et al., 2017). Although, an effective isolation can be accomplished using counter current system. Liu et al. (2016) have applied counter current chromatography for isolation of rutin, quercetin, kaempferol, and daidzein dissolved in choline chloride:glucose:water (2:5:5). Here, ethyl acetate, butanediol, water, hexane or methanol were used in different ratio, and 95.7, 94.6, 97.0, and 96.7% of rutin, quercetin, kaempferol, and daidzein were isolated from the NADES, respectively.

The recovery method for polyphenols from NADES-based extracts nowadays used in the academic circles is solid-liquid extraction with macroporous resins as solid phase. Principle of that method is adsorption of polyphenols from extract on macroporous resin, elution of NADES with water, and desorption of polyphenols with acidified aqueous ethanol (Ruesgas-Ramon et al., 2017). Purification can be performed in batch system or as adsorption chromatography. First, appropriate resin for the polyphenols from plant matrix needs to be selected. Resin selection depends on polarity of extracted polyphenols. For example, ~ 90 % of hydroxysafflor yellow A and cartormin and ~ 84% carthamin were purified with weak polar resin HP-20 (Dai

et al., 2013c). The examples of polyphenols recovery from NADES-extracts by solid-liquid extraction with appropriate resin and recovery efficiency were summarised in paper published by Ruesgas-Ramon et al. (2017). After resin selection, prior to polyphenols adsorption, resin should be washed with 3-5 BV of water followed by 96% (v/v) ethanol and again water since it is packed with salts to avoid contamination. Further, solvents flow through resin, or speed and time of homogenisation in batch cases, concentration of water and acid in ethanol for desorption step should be optimised case-to-case. Final, polyphenols from ethanol fractions could be recovered by evaporation.

Third method that can be found in literature for polyphenols recovery from NADES-extracts is addition of antisolvent. The solubility of NADES in polar solvents (EtOH, water, etc.) makes possible a complete dissociation and solubilization of the NADES forming components. Also, it is well-known that strongly diluted NADES (< 50% of water) it is not NADES, but solution of forming compounds since the characteristic supramolecular interactions between NADES components were broken. Thus, it is possible to extract polyphenols by precipitation or by forming an EtOH- or water- insoluble layer. Removal of EtOH or water solvating NADES components by evaporation allows the recovery of the initial NADES structure (Ruesgas-Ramon et al., 2017). For example, flavonoids from *Flos sophorae* were recovered by adding 20-fold water (antisolvent), and cooled at 0°C, whereupon after 2 h it was possible to recover 75% of rutin by precipitation (Nam et al., 2015).

2.3.Application of NADES in designing a biocatalytic process

When designing a biocatalytic process purity and yield of the product are in the forefront, meaning that is crucial to select a favourable reaction route, biocatalyst, reaction medium and to adjust the parameters and conditions for given system. Furthermore, resource efficiency and sustainability goals need to be met as well (reduction of auxiliary reagents and solvents for each reaction and purification step to reduce amount of waste per unit of product manufactured; selection of auxiliary reagents and solvents with favourable environmental impact) (Wohlgemuth, 2017). The synergistic use of NADES and biotechnological approach biocatalyst fits logically to the efficient and sustainable production of various commercially interesting products. Namely, biocatalysis ensures catalyzing otherwise difficult transformations in high regio-, chemo- and enantioselective manner at mild and economic conditions, whereby NADES can serve as strong green support for modulating/directing reaction route for obtaining the desired product. Possibility to design an optimal NADES for each specific enzymatic reaction

system is important feature since NADES allows to (*i*) enhance enzyme stereoselectivity, activity and stability, as well as reaction rate through enhanced substrate solubility; (*ii*) enhance reaction yield (*iii*) enhance/modify biocatalyst stereo preference;; (*iv*) contribute to overall greenness of the process (including recycle and reuse) (Juneidi et al., 2017; Magdalena Pätzold et al., 2019). In biocatalytic processes NADES can serve as solvent/co-solvent, as extractive reagent for an enzymatic product and pretreatment solvent of enzymatic biomass. Up to now, hydrolases are the most studied enzymes in NADES, which is not surprising giving that lipases are the most important industrial enzymes, while there are limited number of papers dealing with synthetic reactions in NADES involving other hydrolytic enzymes (epoxide hydrolases, phospholipase, proteases and haloalkane dehalogenases), lyases and dehydrogenases (as a part of whole cell biocatalysis) (Table 2.2.). When designing efficient biocatalytic process involving NADES, independently of reaction type and enzyme used, the following steps should be included: (*i*) preparation and characterization of NADES (*ii*) selection of NADES for optimal enzyme performance (*iii*) optimization of the biocatalytic process, and (*iv*) downstream process design with possible scale-up.

Table 2.2. Different reaction types catalysed with isolated enzymes or whole cells in deep eutectic solvents

Screened NADES	Solvent/co-solvent	Biocatalyst	Influence of NADES	Catalyzed reaction	References
ESTERIFICATION					
ChGly* ChU ChEG MetGly	[S]addition of 0-30 % (v/v) of water	*Candida antarctica* B lipase (Novozyme 435)	• the use of water as a cosolvent led to full conversions • in DES with glycerol as HBD used as solvent and substrate	esterification of benzoic acid with glycerol	(Guajardo et al., 2017)
ChGly ChEG* ChU	[S]addition of 0.5–2.5 mol of water, depending on HBD used	*Candida antarctica* lipase B (Novozyme 435)	• esterification yield for all DES tested increased with the increase of water content in DES, however, at certain point optimum was reached and further addition of water negatively impacted the yield	esterification between acetic anhydride and 1-butanol	(Cvjetko Bubalo et al., 2015a)
ChU ChGly* CitGlc FruScu CitFru CitScu CitSol	[S] addition of 0-20% (w/w) of water	*Candida antarctica* lipase B (Novozyme 435)	• with the addition of water into the reaction medium, conversion increased	transesterification between propyl gallate and methanol	(Ülger and Takaç, 2017)
HYDROLYSIS					
ChGlc ChXyl ChXylol ChGly ChEG*	(*i*) [S] with addition of 30 or 50% (w/w) of water (*ii*) [CS] with 80% (w/w) of water	carrot root	• reaction in DES is stereoselective, while in water is not • enantiomeric excess was found to be correlated with water content in DES solution. the highest selectivity is obtained with the lowest content of water in DES	hydrolysis of (±)-1-phenylethyl acetate	(Panić et al., 2017)
• **REDUCTION**					
ChGlc* ChGly* ChEG	(*i*) [S] addition of 30 or 50% (w/w) of water	lyophilised baker's yeast	• increase in conversion with increase water content in NADES	(*i*) reduction of 1-(3-methylphenyl)ethanone (MPA)	(Panić, et al. 2018)

	(*ii*) ^{CS} addition of 80% (w/w) of water		• higher enantiomeric excess in NADES with lower water content	(*ii*) reduction of 1-(3,4-dimethylphenyl)ethanone (DMPA) (*iii*) reduction of 1-(2,4,6-trimethyphenyl)ethanone (TMPA)	
ChGly* ChEG ChOx ChMa ChGlc* ChFru* ChXyl* ChU	^{S} addition of 10, 25 or 50 % (w/w) of water	lyophilised baker's yeast	• increasing the amount of water in NADES beneficially influenced the reaction yield • the higher water content in the reaction medium result better enantiomeric excess	reduction of ethyl 3-oxobutanoate to ethyl 3-hydroxybutanoate	(Cvjetko Bubalo et al., 2015b)
ChGlc* ChXyl ChXylol ChGly ChEG	(*i*) ^{S} with addition of 30 or 50% (w/w) of water (*ii*) ^{CS} with 80% (w/w) of water	carrot root	• inversion of the enantioselectivity by the addition of different amounts of water to NADES • increasing the amount of water in NADES beneficially influenced the reaction yield • reaction yield lower in NADES than in water	reduction of 1-(3,4-dimethylphenyl)ethanone (DMPA)	(Panić et al., 2017)
ChGly	(*i*) ^{S} with addition of 0, 5, 10, 20 or 40 % (w/w) of media (*ii*)^{CS} with addition of 60, 80, 90 or 95 % (w/w) of media	*Ralstonia* sp. alcohol dehydrogenase (ADH), *Thermoanaerobacte*r ADH and horse liver ADH overexpressed in *Escherichia coli*	• the enantiomeric excesses obtained for abroad range of aromaticsubstrates increase if NADES is added • decreasing of reaction yield with increasing the percentage of NADES in aqueous media	reduction of 2-octanone, benzaldehyde, or propiophenone	(Müller et al., 2015)

ChGly ChEG* ChU	[CS]with addition of 95 % (v/v) of potassium phosphate buffe	recombinant whole-cells containing AKRs and glucose dehydrogenase (GDH)	• the whole cell activity dependent on HBD	reduction of *N*-ethyl-*N*- methyl carbomyl acetophenone	(Xie et al., 2019)
DEHYDROGENATION					
ChGly ChU* ChEG	[CS]with addition of 96 % (v/v) of cell suspension	whole cells of *Arthrobacter simplex*	• higher initial reaction rates than those conducted in the presence of the NADES's individual components • increased solubility of the substrate in NADES	1,2-dehydrogeneration of cortisone acetate	(Mao et al., 2018)
OXIDATION					
ChGly	[CS] with addition of 90% (v/v) of buffer in two-phase system with [C4MIM][PF6]	*Acetobacter* sp. CCTCC M209061	• increased solubility of the substrate with NADES addition, reducing the toxic and inhibitory effects of the substrate • decreasing the reaction time, higher initial reaction rate	asymmetric oxidation of 1-(4-methoxyphenyl) ethanol	(Wei et al., 2016)

*optimal solvent for the highest reaction yield
CS-cosolvent
S-solvent
Component abbreviations: Ch, choline chloride; Cit, citric acid; CL, dried cellulose hydrolysate fraction (71.6 % Glc and 16.6 % Xyl); DEAC, N,N-diethyl ethanol ammonium chloride; DEG, diethylene Glycol; EAC, ethylammonium chloride; EG, ethylene glycol; Fru, fructose; Glc, glucose; Gly, glycerol; Ma, malic acid; Met, methylammonium chloride; Mo, malonic acid; Ox, oxalic acid Sol, sorbitol; Scu, sucrose; TEC, Triethylene Glycol; U, urea; Xyl, xylose; Xylol, xylitol.

2.3.1. Selection of NADES for optimal enzyme performance

Previous research has indubitably demonstrated that by selecting an appropriate DES with an optimal chemical nature (their compositions with different salts and HBD and the molar ratios between the two) and water content, an enzyme can be highly stabilized and activated. The composition of NADES also is directly connected to the solubility of other reaction participants (substrates and products), thus directly in correlation to reaction rate, yield and complexity of product isolation and recovery. Therefore, for each enzyme/substrate systems, a comprehensive screening of NADES should be applied.

Searching the literature, it is evident that commonly used NADES in biocatalysis are choline chloride-based ones containing HBD either sugar, sugar alcohols, amide or polyalcohols (Table 2.1.). In some studies, NADES containing organic acids was also considered, but poor biocatalyst activity was observed (Magdalena Pätzold et al., 2019; Cvjetko Bubalo et al., 2015b). Knowing that biocatalysis is usually performed at mild operational and environment-friendly conditions this is not surprising. However, sometimes choosing more desirable HBD it is not so evident as is it case for urea-based NADES as one of the first and the most studied one. Urea is widely known as a natural agent to cause denaturation of proteins while choline chloride:urea possess pH value more than 8 on room temperature. Nevertheless, choline chloride:urea in many studies was proven to be the most favourable medium for biocatalysis, indicating that some other factors are dominant in predicting process efficiency. Probably, key issues lay in the hydrogen bonding within DES and reaction participants (substrate, product and biocatalyst). For example, the formation of hydrogen bonds between urea and choline and chloride ions in DES prevents diffusion of urea into the protein core and hinder urea attack on the enzyme functional domains thus resulting in stabilized enzymes (Monhemi et al., 2014).

Accordingly, the selection of optimal NADES is performed case to case scenario. It was also observed that molar ratio can significantly affect the enzyme activity. Different molar ratios of the HBA and HDB might change hydrogen bonding formation lead to alteration in previously mentioned effect of H-bond net of reaction efficiency.

H-bond net could also have great impact on solubility of substrate/product. In general, hydrogen bonding in responsible for remarkable better solubility of both polar and nonpolar compound which could strongly influence biocatalysis efficiency (Mao et al., 2018; Magdalena Pätzold et al., 2019). More specifically, our research group noticed that substrates can be incorporated in NADES with H-bond net which could pose a serious problem for a variety of enzyme-catalyzed processes and should be handled individually, depending on both enzyme

and reaction type (sometimes a simple addition of water proved to be effective enough). On the other hand, the possibility of product entrapment in the same H-bond net could be very interesting from a point of chemical equilibrium, since the product formed could be virtually removed from the enzyme surrounding, shifting the chemical equilibrium towards product formation (Cvjetko Bubalo et al., 2015a).

Moreover, when selecting NADES for whole cell biocatalysts especially when regeneration of the coenzyme presents in the cells some additional criteria should be taken in account. For instance, HBD (sugars and alcohols) can play an important role as co-substrates for cofactor regeneration or as a nutrition source for living cells as biocatalysts in various bioreduction reactions and consequently this NADES could be a good selection (Cvjetko Bubalo et al., 2015b). Indeed, higher bioreduction conversions obtained in medium containing sugar as HBD suggest that the presence of auxiliary substrates (e.g. sugar) for cofactor-recycling system which could reduce the problem of co-factor consumption and, therefore, greatly enhance the bioconversion (Mbous et al., 2017). Also, NADES composition could influence reaction stereoselectivity significantly performing whole-cell biocatalysis. When studied reduction of 1-(3,4-dimethylphenyl)ethanone (DMPA) by *S. cerevisiae*, Panić et al. (2018) observed that by using choline chloride-based NADES with glucose, ethylene glycol or glycerol as HBD, enantiomeric excess (*ee*) varied from 63.1 to 86.7 % in favour of *S*-alcohol.

Adjusting water content in NADES is crucial since the water possess detrimental effect on NADES physiochemical characteristic. Indeed, majority of investigated biocatalytic processes are in NADES are governed in the presence of water, therefore it is crucial to understand the role of water in such complex mixture. Water can significantly reduce NADES viscosity, influence chemical equilibrium (especially in hydrolysis/synthesis) reactions or change the enzyme activity/stability (Cvjetko Bubalo et al., 2015b; Mitar et al., 2019). However, it is difficult to predict how the molecules involved in this network (components of NADES and water) are organized, and it is equally difficult to predict their respective influence on the course of enzyme-catalyzed reactions.

Understanding the mechanisms of how the NADES can influence the activity and stability of enzyme, as well as interaction of the NADES with other reaction participants (e.g. substrate, co-substrate and product) is vital for future progresses in the field of NADES-assisted biocatalysis. Gorke et al. (2010) were the first to observe that the components within the NADES are 20- to 4600-fold less reactive than expected based on their concentration in lipase-catalyzed transesterification reactions, implying that hydrogen-bond network in NADES lowers the chemical potential of NADES components. Later, Huang et al. (2014) and Zhao et al. (2015)

confirmed that pairing anions such as chloride and acetate with various HBD might weaken the reactivity of enzyme-denaturing or HDBs as well as urea.

2.3.2. Optimization of biocatalytic process within NADES

Adjusting the process parameters to achieve the maximum economic efficiency of the biocatalytic process within NADES is carried out as every other biocatalytic process. This means optimization of substrate/enzyme loading, pH, temperature, reaction time, and implementation of some techniques such as enzyme immobilization, development of two-phase systems, enzyme/cell pretreatment, cofactor application and development of cascade systems and chemoenzymatic routes. Choosing the right NADES for specific biocatalytic system is also a major part of biocatalytic process optimization since it directly influences pH, polarity and viscosity of a medium, and as such directly influence the enzyme performance, substrate/product solubility (Paiva et al., 2014).

It is well known that raising temperature up to temperature optimum speeds up enzyme-catalyzed reactions, whereas high temperatures (depending of enzyme origin and type) cause an enzyme to lose its active shape. As already mentioned, adjusting reaction temperature in NADES-mediated biocatalytic reactions is especially important as elevation of temperature reduces viscosity of NADES, enabling better mass transfer in such viscous environment and consequently higher reaction rate. For instance, Abbott et al. (2004) showed that the viscosity of NADES changes considerably in function of the temperature. For example, for NADES choline chloride:urea an increase of the temperature from 20°C to 50°C, caused 10-fold decrease of the viscosity of the solvent. Accordingly, Durand et al. (2012) showed that initial specific activity of *Candida antarctica* lipase B is 1.5 times higher when the temperature increases by 20°C (from 40°C to 60°C) in DES choline chloride:urea or choline chloride:glycerol.

Furthermore, from the literature it is evident that variation in pH value influences the reaction rate and yield through the activity and selectivity of the enzymes, as well as the regeneration of the coenzyme present in the cells. The pH value influences the ionic state of the substrate and enzyme thus leading to changes in enzyme's activity and enantioselectivity. This is especially interesting when the substrate could be transformed by several isoenzymes with different enantioselectivity at different pH (Panić et al., 2017). Environment of neutral pH is usually optimal for the most of biocatalytic reactions, therefore it is expected that pH neutral

NADES should be the best option for performing transformations (Cvjetko Bubalo et al., 2015b; Maugeri and Domínguez De María, 2014).

As substrate solubility and loading is one of the key parameters influencing on biocatalytic process productivity, several research groups investigated difference in optimal substrate concentration in NADES compared to referent solvents (buffer or organic solvents). Studies implied that NADES-containing system can bring a remarkable improvement in the reaction efficiency in respects of substrate solubility/loading and consequently improved productivity of biocatalytic process (Mao et al., 2018; Xu et al., 2016, 2015). For example, NADES used as co-solvents enhanced the efficiency of C1,2-dehydrogenation by improving the solubility of the highly hydrophobic substrate (Mao et al., 2018). To be more precise, the solubility of cortisone acetate in choline chloride:urea was improved to 0.0436 mg mL^{-1}, in comparison to solubility in water (0.0215 mg mL^{-1}) and EtOH (0.0280 mg ml^{-1}). The fact that enzymes tolerate high concentrations of substrates in the presence of NADES, together with the fact that pull of possible raw materials for NADES preparation is enormous, promoted the idea of incorporating substrates in a NADES matrix to provide high substrate loads. Using substrate-based NADES as reaction media was exploited for a number of different enzymatic reactions. For these reactions, one of the substrates was incorporated in the NADES matrix, whereas it was also possible to form NADES with both substrates for solvent-free lipase catalyzed esterification reactions (Guajardo et al., 2017; Pöhnlein et al., 2015; Ranganathan et al., 2017; Siebenhaller et al., 2016; Zeng et al., 2015). Recent paper of Pätzold et al. (2019) reported *Candida rugosa* lipase catalyzed esterification of (-)-menthol and lauric acid in a NADES mixture formed by both substrates ((-)-menthol:lauric acid 3:1, mol mol^{-1}), whereby no additional reaction solvent was necessary since the DES acts as combined reaction medium and substrate pool (95% lauric acid conversion was achieved). The authors pointed out that the importance of this achievement lies in the several facts: enabled high substrate concentrations, reduction of waste and easier product recovery. Interesting approach was reported by Ranganathan et al. (2017) who develop a novel NADES mixture that could act as both the solvent and the co-substrate source in fast and efficient epoxidations.

Furthermore, immobilization of biocatalyst, as well as development of biphasic system, are techniques that could lead to better process efficiency, enable easier isolation of product or biocatalyst/solvent recovery, or to decrease inhibitory effect of substrate/product and to shift reaction equilibrium toward product formation (in case of a biphasic system). Interesting examples for immobilization of biocatalyst when running the reactions within NADES-containing systems was presented by Andler et al. (2017). Andler et al. (2017) demonstrated

improved enzyme (lipase B from *Candida antarctica*) performance in terms of activity and stability, together with process sustainability, in green solvent systems (NADES) via hierarchical assembly (systems that integrate nano- and macroscale structural elements can offer enhanced enzyme stability over traditional immobilization methods).

2.3.3. Downstream process design with possible scale up

Downstream separation of target product from NADES in biocatalytic process is challenge and should be developed for every specific case. In case of biocatalysis, downstream processing involves the removal of the biocatalyst from reaction media and the isolation and purification of final product (with desirable recyclation of biocatalyst/solvent) (Woodley, 2008). Biocatalyst can be relatively easily removed with sedimentation, centrifugation or filtration (especially if immobilized biocatalyst is used), while product isolation is a big challenge. When performing biocatalysis in organic solvent, solvent is usually evaporated and final product is further purified. On the other hand, low vapour pressure of NADES makes these solvents very difficult to evaporate, which may be a problem for industrial applications (Cvjetko Bubalo et al., 2015c). Only a few research groups have reported a downstream separation of product after biocatalytic process in NADES (Jiang et al., 2019; Maugeri et al., 2012; Panić et al., 2018). Panić et al. (2018) recovered product (*S*)-1-(3,4-dimethylphenyl)ethanol (obtained by *S. cerevisiae*-mediated reduction of corresponding acetophenone) from reaction media by liquid-liquid extraction using agro-solvent ethyl acetate. Maugeri et al. (2012) separated alcohol from ester with DES whereas ester formed second phase. This could be a good choice of recovery and recyclation if the product or substrate are not soluble in NADES. If the product is volatile, distillation could be used, as demonstrated by Jiang et al. (2019). Specific resins for selective adsorption of product can be also applied (Dafoe and Daugulis, 2014). Important thing to have in mind with product recovery is that NADES form hydrogen bonds with products and substrates, so prior to recovery, the bonds between NADES and target compounds should be broken with water addition in case of hydrophilic NADES since the presence of >50 % (v/v) of water ruptures the NADES structure (Cvjetko Bubalo et al., 2015a; Panić et al., 2019).

The ability to recycle the participants of the reaction (biocatalyst and solvent) is also important from the efficiency and sustainability point of view. Therefore, several groups have reported successful recyclation of cells and NADES. Mao et al. (2015) demonstrated the reusability of recycled immobilized *A. simplex* and NADES in bio dehydrogenation of steroid. Recycling of the immobilized cells was performed by direct filtration of the reaction medium, and then the choline chloride:urea-containing system was recovered by simple biphasic

extraction with ethyl acetate in order to eliminate the substrate and the product (the process was performed for five cycles and decrease in substrate conversion from 93% to 81% after the fifth cycle was observed). When transformation of isoeugenol to vanillin in DES choline chloride:galactose. Yang et al. (2017) successfully recycled immobilized *Lysinibacillus fusiformis* CGMCC1347 cells, whereby the immobilized cells also exhibited an excellent operational stability: their catalytic activity was well maintained for at least 13 cycles (72 h reaction for each cycle), after which the reaction was terminated. To estimate the reusability of the *Acetobacter* sp. CCTCC M209061 cells, Xu et al. (2015) investigated reuse of the cells was in the chloride:urea-containing reaction system under the optimized reaction conditions. The immobilized cells showed superior retention of activity in NADES-based system compared to that in aqueous buffer (after 5 batches the biocatalyst retained nearly 80.0% and 50.4% of its initial activity in system with DES and buffer, respectively). Panić et al. (2018) recycled and reuse DES after performing baker's yeast catalysed asymmetric reduction of acetophenone in choline chloride:glycerol. After product recovery by ethyl acetate, the DES were successfully used twice in the asymmetric reduction. Reduction yield was slightly reduced in each cycle from 89.91% to 82.76% and 87.53% during the second and third cycles, respectively. A slight decline in the *ee* value from 88.52 % in first cycle was also observed in the second and third cycles (84.16% and 77.72%, respectively). The yield of the NADES recycled once and twice was 73.33% and 58.33%, respectively, of the freshly synthesised solvent in first cycle (100%).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

- 0.25% Tripsin-EDTA, GIBCO Invitrogen Corporation, United Kingdom
- 2,2′-Azobis(2-methylpropionamidine) dihydrochloride, Acros Organics, USA
- (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Aldrich, Germany
- Acetonitrile (99.8%), J.T. Baker, Netherlands
- Betaine, 98%, Acros Organics, USA
- Catechin, Sigma-Aldrich, USA
- CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS test), Promega Corporation, USA
- Choline chloride (99 %), Acros Organics, USA
- Citric acid, Gram-mol d.o.o., Croatia
- Cyanidin-3-(6-*O*-p-coumaroyl)monoglucosides, Sigma-Aldrich, USA
- D,L-malic acid ≥ 98%, Sigma-Aldrich, USA
- Delphinidin-3-*O*-monoglucoside, Polypolyphenols AS, Norway
- Disodium phosphate, Kemika, Croatia
- Distilled water, Faculty of Food Technology and Biotechnology, Croatia
- DMEM (*Dulbecco's Modified Eagle's Medium*), GIBCO Invitrogen Corporation, Paisley, United Kingdom
- DMSO d_6, Sigma-Aldrich, USA
- Ethanol (96%), Kemika, Croatia
- Ethyl acetate, Sigma-Aldrich, USA
- Ethylene glycol, (>99%), Fisher Scientific, United Kingdom
- FBS (*Fetal Bovine Serum*), GIBCO Invitrogen Corporation, New Zeland
- Fluorescein, Sigma-Aldrich, USA
- Folin ciocalteau reagent, Kemika, Croatia
- Formic acid (98-100%), T.T.T., Croatia
- Fructose, 99,99%, Lach-Ner, Czech Republic
- Galic acid, Sigma-Aldrich Co., USA
- Glucose, >95%, Acros Organics, USA

- Glycerol, 95%, Kemika, Croatia
- Hydrochloric acid, Kemika, Croatia
- L-proline 98,5% Sigma-Aldrich, USA
- Malvidin-3-(6-*O*-p-coumaroyl)monoglucosides, Polypolyphenols AS, Norway
- Malvidin-3-acetylmonoglucosides, Polypolyphenols AS, Norway
- Malvidin-3-*O*-monoglucoside, Polypolyphenols AS, Norway
- *n*-heptane, J.T. Baker, Netherlands
- The CellTiter 96® AQ_{ueous} One Solution Assay {MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium}, Promega, USA
- Muse™ Oxidative Stress Kit, Millipore, Merck KGaA, Germany
- Muse™ Annexin V & Dead Cell Kit, Millipore, Merck KGaA, Germany
- Muse™ Cell Cycle Kit, Millipore, Merck KGaA, Germany
- *n*-heptane, Sigma-Aldrich, USA
- Nile red, Sigma-Aldrich, USA
- Peonidin -3-(6-*O*-p-coumaroyl)monoglucosides, Polypolyphenols AS, Norway
- Peonidin-3-acetylmonoglucosides, Polypolyphenols AS, Norway
- Peonidin-3-*O*-monoglucoside, Polypolyphenols AS, Norway
- Potassium chloride, Kemika, Croatia
- Potassium dihydrogen phosphate, Kemika, Croatia
- Quercetin-3-*O*-glucoside, Sigma-Aldrich Co., USA
- (*R*)-1-phenylethanol, Sigma–Aldrich, Germany (purity of ≥99%)
- (*R,S*)-1-phenylethyl acetate, Sigma–Aldrich, Germany (purity of ≥99%)
- (*S*)-1-phenylethanol, Sigma–Aldrich, Germany (purity of ≥99%)
- Sepabeads SP825L macroporous resin, Tecno Impianti Srl, Pozzuolo M.na – Milan, Italy
- Sodium chloride, Kemika, Croatia
- Sodium dihydrogen phosphate dihydrate, Kemika, Croatia
- Sodium hydrogen carbonate, Kemika, Croatia
- Sodium hydrogen sulfate, Sigma-Aldrich Co., USA
- Sodium hydroxide, Kemika, Croatia
- Sorbose, Sigma-Aldrich Co., USA
- Tripan-blue, Sigma-Aldrich, USA

- Trypsin-EDTA solution, Sigma-Aldrich, USA

3.1.2. Enzyme

Novozym 435 (lipase B from Candida antarctica; immobilizedon macro-porous polyacrylic resin beads, > 10 000 U g^{-1}) was obtained from Novozymes (Bagsvard, Denmark).

3.1.3. HeLa, MCF-7 and HEK cell lines

In this work, two human tumour cell lines (HeLa and MCF-7) and one human cell line (HEK293T) were used. Cell lines were obtained from the Ruđer Bošković Institute (Zagreb, Croatia).

HeLa cell line derived from the cervical adenocarcinoma (ATCC No. CCL-2™), MCF-7 derived from metastatic site: pleural effusion mammary gland, breast (ATCC® HTB-22™) and HEK293T cell line derived from embryonic kidney (ATCC No. CRL-3216™) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and maintained in T-flaks in the incubator with humidified atmosphere and 5% CO_2 at 37°C. Individual experiments to test cytotoxicity of NADES and grape pomace extracts were performed in 96-wells plates.

3.1.4. Biomass

Grape pomace was obtained from the Croatian native red grape cultivar, *Vitis vinifera* cv. Plavac mali, which originates from Dalmatia (Croatia southern vine-growing region). It was freeze-dried (Alpha 1–2 LD plus Christ, Germany) for two days, milled and stored at 25°C in a desiccator until extract preparation.

3.1.5. Buffers and solutions

Phosphate buffer (0.2 M, pH=7)

Sodium dihydrogen phosphate dihydrate (6.242 g and 200 mL distilled water)		39 mL
Disodium phosphate (5.687 g and 200 mL distilled water)		61 mL
Distilled water	until	200 mL

AAPH solution (2,2′-azobis(2-methylpropionamidine) dihydrochloride)

AAPH		0.207 g
Phosphate buffer (0.075 M)	until	5 mL

Fluorescein solution

Stock solution 1: 15 mg fluorescein in 100 mL phosphate buffer (0.075 M)

Stock solution 2: 100 µL stock solution 1 in 10 mL phosphate buffer (0.075 M)

Stock solution 3: 50 µL stock solution 2 in 50 mL phosphate buffer (0.075 M)

PBS buffer (pH=7.4)	
Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate	1.44g
Potassium dihydrogen phosphate	0.24g
Distilled water	1000 mL
0.4% Trypan blue solution	
Trypan blue	0.04 g
PBS buffer	10 mL

3.1.6. Apparatus and accessories

- Agilent 1200 Series HPLC system (Agilent, San Jose, CA, USA) equipped with a diode array detector (DAD)
- Analytical balance, BAS 31 plus, BOECO, Germany
- AS 1000- Automatic environmental SpeedVac, Savant, USA
- Autoclave A-63C, Kambič, Slovenia
- Automatic double column system, STF, University of Turin and Tecnoimpianti Skid, Italy
- Bruker AV-300 spectrometer at NMR Centre on Ruđer Bošković Institute (Zagreb, Croatia)
- Centrifuge D-78532, Hettich Zentrifugen, Njemačka
- Centrifuge ROTOFIX 32, Hettich Zentrifugen, Germany
- Eppendorf ThermoMixer C, Eppendorf, Germany
- Homogenizer/incubator ES-20/60, Biosan, Latvia
- IKA vortex GENIUS 3, Sigma-Aldrich, USA
- Inverted microscope, Zeiss, Germany
- Laboratory balance BAS 31 plus, Boeco, Njemačka
- Light microscope Axiostar 1122-100, Carl Zeiss, Njemačka
- Magnetic stirrer, MM-510, Technica Železnik, Slovenia
- Microplate reader, Tecan, Mannedorf, Switzerland
- Muse® Cell Analyzer, EMD Milipore Corporation, USA
- MW-US-ER-01 cooperative extractor/ reactor (LAB-KITS, China)

- pH electrode InLab Expert Pro-ISM, Mettler Toledo, USA
- pH/ion meter S220, Mettler Toledo, USA
- Rheometer Scientific RM180, Switzerland
- Rotacioni-vakuum uparivač, B-485, Buchi Oilbath, Švicarska
- Savant SPD131DDA SpeedVac Concentrator, Thermo scientific, USA
- Shimadzu gas chromatograph QP2010PLUS instrument equiped with MS detector, Japan
- Spectrophtometer GENESYS 10S UV-Vi, Thermo Scientific™, USA
- SynthWAVE reactor (Milestone/MLS), Italy
- US bath XUB5 (XUB Series Digital Ultrasonic Baths, BioSan, Latvia), equipped with Digital LCD controls, a timer and a heater (Heater power 150 W)
- US converter, University of Turin, Italy
- US generator, University of Turin, Italy
- US Titanium horn prototype, University of Turin, Italy
- Varian Cary Eclipse Spectrofluorimeter, USA
- Vertical Laminar Air Flow Cabinet, Kambič, Slovenia

3.1.7. Software packages

Data analysis software system STATISTICA version 8.0. (StatSoft Inc. 2007, www.stasoft.com) was used for statistical data analysis.

The software Design-Expert version 7.0 (State-Ease, USA) was used for calculation and fitting the values of the independent variables to the proposed models.

Chem Draw Ultra 12.0.2.1076 (CambridgeSoft) was used for creation of chemical structures and reactions.

Mestre Nova 6.0.2.-5475 (Mestrelab Research S.L.) was used as 'Data Analysis' feature for the analysis of an array of ^{1}H NMR experiments.

3.2. Methods

3.2.1. Preparation of selected natural deep eutectic solvents

Choline chloride (ChCl) was dried in a vacuum concentrator (Savant SPD131DDA SpeedVac Concentrator, Thermo scientific, USA) at 60°C for 24 h before use. The NADES were prepared at certain molar ratios of ChCl to hydrogen bond donor (HBD). The two or more components were placed in specific ratio, with 10 - 50 % (v/v) of water, in a round-bottomed

glass flask, and then were stirred and heated to 50°C for 2 hours until a homogeneous transparent colourless liquid was formed. NADES abbreviations and corresponding mole ratios are given in Table 3.1.

Table 3.1. Used NADES

Natural deep eutectic solvents	Abbreviation	Molar ratio
Choline chloride:citric acid	ChCit	2:1
Choline chloride:malic acid	ChMa	1:1
Choline chloride:proline:malic acid	ChProMa	1:1:1
Proline:malic acid	ProMa	1:1
Betaine:malic acid	BMa	1:1
Betaine:citric acid	BCit	1:1
Malic acid:glucose:glycerol	MaGlcGly	1:1:1
Malic acid:glucose	MaGlc	1:1
Choline chloride:glucose	ChGlc	1:1
Choline chloride:glycerol	ChGly	1:2
Choline chloride:ethylene glycol	ChEG	1:2
Glucose:glycerol	GlcGly	1:2
Glucose:ethylene glycol	GlcEG	1:2
Sorbose:ethylene glycol	SorEG	1:2
Ethylene glycol:glucose:fructose	EGGlcFru	2:1:1

3.2.2. Physicochemical characterisation of NADES

The pH values for each NADES were determined using a 405-DPAS pH-electrode (Mettler Toledo, Zagreb). The pH measuring range was 0–12 in a temperature range of 0–100°C.

The polarity of each NADES was determined using Nile red as a solvatochromic probe, as described in Jeong et al. (2017) and Ogihara et al. (2004). Briefly, a Nile red solution (1.0 g

L^{-1}) was prepared and diluted 100 times in 96% (v/v) ethanol, and then added to the NADES, after which the absorption spectra of the dye (λ_{max}) were measured using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The molar transition energy (E_{NR}) was calculated using the following formula (Reichardt, 1994):

$$E_{NR}(kcal\ mol^{-1}) = \frac{h*c*N_A}{\lambda_{max}} \quad [3.1]$$

where λ_{max} is maximum absorption wavelength, h is Planck's constant, c is the velocity of light and N_A is Avogadro's constant.

The viscosities of investigated NADES were determined using a Rheometric Viscometer (Model RM 180, Rheometric Scientific, Inc., Piscataway, USA) with the spindle (no. 3; Ø = 14 mm; l = 21 cm). Shear stress against the increasing shear rates from the lowest value of 0 s^{-1} to 1290 s^{-1}, as well as downwards, was applied. Measurements were done in triplicates for each sample. The shear rate versus shear stress was interpreted using the Rheometric computer program. The values for n and k were obtained from plots of log shear stress versus log shear rate, according to the power law equation:

$$\log \tau = \log k + \log \gamma \quad [3.2.]$$

where τ is the shear stress (Pa); γ is the shear rate (s^{-1}); n is the flow behavior index, and k is the consistency coefficient (Pa s^n).

$$\tau = \eta_{app}\gamma \quad [3.3.]$$

Apparent viscosity (η_{app}) was calculated at 1290 s^{-1} using Newtonian law, in addition to linear least square method for regression analysis.

3.2.3. Evaluation of biological properties of NADES

The influence of synthesized NADES on cell proliferation was examined by CellTiter 96® AQ_{ueous} One Solution Assay for colorimetric analysis, as described in Mitar et. al (2019). Briefly, HEK-293T cells at initial concentration of $5x10^4$ cells mL^{-1}, HeLa and MCF-7 cells at initial concentration of $3x10^4$ cells mL^{-1} were seeded in 96-well plates. After 24 hours of growth, cells were treated with three different nominal concentrations (500 mg L^{-1}, 1000 mg L^{-1} and 2000 mg L^{-1}) of prepared NADES, filtered through 0.22 μm filters, in four parallels. After 72 hours of treatment, 10 μL of CellTiter 96® AQueous One Solution reagent, a novel tetrazolium compound [3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)], was added to each well. The cells were

further treated for 4 h, when MTS reagent was bioreduced by living cells into a coloured formazan. The quantity of formazan product, which is soluble in culture medium, was measured by absorbance at 490 nm on the microplate reader (Tecan, Switzerland) and was directly proportional to the number of viable cells in culture. Cell viability was calculated as described in (Radošević et al., 2016c). Expressed data are the mean values of experiments performed three times.

Oxygen radical absorbance capacity (ORAC) assay was performed based on the method described by Mitar et al. (2019) and the results were expressed as relative ORAC values (eq. 3.2.). The assay was conducted in a quartz stones with 3 mL of reaction mixture with 2.25 mL of fluorescein sodium salt (0.04 μmol L^{-1}) in sodium phosphate buffer (0.075 M, pH 7.0), and 0.375 mL diluted NADES, Trolox (25 μmol L^{-1}) as standard or 0.075 M sodium phosphate buffer (pH 7) as blank control. After incubation for 30 min at 37°C 0.375 mL of AAPH was added. Fluorescence was recorded every minute up to value zero by a Varian Cary Eclipse Spectrofluorimeter (Palo Alto, CA, USA) with 485 nm excitation and 520 nm emission. Results were analysed using the differences of areas under fluorescein decay curve between the blank and the sample. The results were the mean values (n=3) and were expressed as μmol Trolox equivalent per g of NADES (μmol TE g^{-1}).

Relative ORAC value (μmol TE g^{-1}) was calculated according to eq (3.4.) :

$$\text{relative ORAC-value} = \left(\frac{AUC_S - AUC_B}{AUC_{TRX} - AUC_B}\right) \times k \times \alpha \times h \qquad [3.4.]$$

where, AUC_S is the area under the curve of sample, AUC_B is area under the curve of blank, AUC_{TRX} is area under the curve of Trolox, *k* is dilution factor, *α* is molar concentration of Trolox, and h is ratio of volume and mas of the sample.

3.2.4. Methodology for development of an environmentally friendly method for isolation of anthocyanins from grape pomace using NADES

3.2.4.1. Selection of optimal NADES

Extraction was performed in a US bath XUB5 (XUB Series Digital Ultrasonic Baths, BioSan, Latvia), equipped with Digital LCD controls, a timer and a heater (heater power 150 W). All extractions were carried out under US (power 100 W) at constant temperature (65°C) for 50 min. Solid-liquid ratios of 0.03 g of freeze-dried grape pomace per mL of prepared NADES, which contained 25% (v/v) of water (ChCit, ChMa, ChProMa, ProMa, BMa, BCit,

MaGlcGly and MaGlc) or acidified aqueous ethanol (70% of ethanol with 0.1% of HCl, v/v), were used for extraction. Extracts were then centrifuged for 15 min at 5000 g, the supernatant was decanted, adjusted to a final volume of 10 mL (0.03 mg mL^{-1}) and stored at +4°C until further analyses were performed (paragraph 3.2.2.3.).

3.2.4.2. Testing the stability of prepared extracts

The storage stability of the prepared extracts was monitored according to anthocyanins degradation as described in the procedure reported by Dai et al. (2014). Extracts were stored in the dark at 25°C, 4°C and -18°C for 60 days, and were monitored and analysed over the 60 days using HPLC (paragraph 3.2.2.3.). Data are expressed as degradation rate (c/c_0).
Degradation rate (w/w_0, %) is calculated according to eq. 3.5.:

$$Degradation\ rate = \frac{w}{w_0} \quad [3.5.]$$

where w_0 is the content of anthocyanins (mg g_{dw}^{-1}) and w is the content of anthocyanins (mg g_{dw}^{-1}) after incubation.

3.2.4.3.Analyses of prepared extracts

Determination of total anthocyanin content

The content of total anthocyanin (TA) in grape pomace extracts was determined by the bisulfite bleaching procedure (Ribereau-Gayon & Stonestreet, 1965), since anthocyanins form colorless compounds with bisulfite ion, transforming from red-colored flavylium cation to non-colored form. The differences obtained between the nonbleached and bisulfite-bleached sample in absorbance at 520 nm are proportional to the total anthocyanin content. Total anthocyanin content is expressed as mg of malvidin-3-*O*-glucoside equivalents per g of pomace (mg g^{-1}). Spectrophotometric analyses were conducted in triplicate.

Determination of total phenolic content

Total phenolic content (TP) was determined by Folin-Cicalteu method as briefly described in (Singleton et al., 1999). The absorbance was measured at 760 nm and results were expressed as mg of gallic acid equivalent per g of grape pomace (mg_{GAE} g^{-1}). Spectrophotometric analyses were conducted in triplicate.

HPLC analyses

HPLC analyses were performed on the Agilent 1200 Series HPLC system (Agilent, San Jose, CA, USA) equipped with a diode array detector (DAD) and Phenomenex C18 column

(Kinetex 150 mm × 4.6 mm, 2.6 µm, 100 Å). Prior to analysis, samples were filtered through 0.22 µm PTFE (polytetrafluoroethylene) filters.

Mobile phases for anthocyanins analyses were water/formic acid (95:5, v/v) (solvent A) and acetonitrile/formic acid (95:5, v/v) (solvent B). Analyses were performed with the following elution gradient conditions, according to solvent B: 0-15.5 min, 10-35%; 15.5-16 min, 35%-100%, 16-17 min, 100%, 17-18 min, 100%-10%. Flow rate was 0.9 mg mL^{-1}. The column temperature was 40°C, and the autosampler temperature 4°C. Anthocyanins (delphinidin-3-*O*-monoglucoside, petunidin-3-*O*-monoglucoside, peonidin-3-*O*-monoglucoside, malvidin-3-*O*-monoglucoside, peonidin-3-acetylmonoglucosides, malvidin-3-acetylmonoglucosides, cyanidin-3-(6-*O*-p-coumaroyl)monoglucosides, peonidin -3-(6-*O*-p-coumaroyl)monoglucosides and malvidin-3-(6-*O*-p-coumaroyl)monoglucosides) were detected and identified comparing their retention times and spectral data with authentic external standards, and were quantified with external standard calibration curve (3-300 mg L^{-1}) of malvidin-3-*O*-monoglucoside, at the wavelength of maximum absorbance (520 nm).

Mobile phases for the separation of polyphenols were water/formic acid (99.9:0.1, v/v) (solvent A) and methanol/formic acid (99.9:0.1, v/v) (solvent B). and analysed by a HPLC with following elution gradient according to solvent B: 0–3 min, 10%; 3–15 min, 10%–50%; 15–20 min, 50%-60%; 20–25 min, 60%-100%; 25-26 min 100%. Flow rate was 0.9 mL min^{-1}, The column temperature was kept at 30°C and the autosampler at 4°C. The retention times and spectral data of polyphenolic compounds were compared with external standards. Gallic acid and catehin were identified at 280 nm and quercetin-3-*O*-glucoside at 360 nm. Polyphenols were quantified considering calibration curves of authentic external standards (3-300 mg L^{-1}) at the wavelength of maximum absorbance.

HPLC analyses were conducted in triplicate. Content of anthocyanins and polyphenols were expressed as mg of compound per g of dry weight (dw).

3.2.4.4.Extraction method selection and optimization

The influence of US and MW, used alone and simultaneously, on extraction efficiency was investigated. Experiments were performed in a MW-US-ER-01 cooperative extractor/ reactor (LAB-KITS, China) for 10 min with US (40 kHz, 50 W) and/or MW (100 W) irradiation. 0.3 g of grape pomace was extracted with 10 mL of ChCit (25%, v/v). Extracts were then centrifuged and the supernatant was stored and total anthocyanins content was determined as described in paragraph 3.2.2.3.

The optimization of the UMAE of grape-pomace anthocyanins was performed by using response surface methodology (RSM) via Box-Behnken design. The influence of the independent variables, MW power (X_1, 100–300 W), water content in NADES (X_2, 10–50%, v/v) and extraction time (X_3, 3–15 min), on the dependant variable, extracted anthocyanin content (Y), was investigated (Table 3.2.). US power was constant (50W) in all preformed experiments due to instrument set up, and the maximum temperature was 80°C. Fifteen experiments were performed with 3 centre points per block to optimise the extraction method, and the responses were fitted with a second-order polynomial equation (RSM model) (Fernández et al., 2018).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad i < j \qquad [3.6.]$$

where X_1, X_2,. . ., X_k are the independent variables, Y the dependant variable, β_0, β_j (i = 1, 2,. . ., k), β_{ii} (i = 1, 2,. . ., k), and β_{ij} (i = 1, 2,. . ., k; j = 1, 2,. . ., k) are the regression coefficients for the intercept, linear, quadratic and interaction terms, respectively, and k is the number of variables.

Design-Expert (Version 7.0.0., Suite 480, Minneapolis, MN 55413) software was used for the analysis of variance (ANOVA) to obtain the quadratic polynomial mathematical model, which was established to describe the interaction of process parameters on extraction of total anthocyanins. The value of the determination (R^2) and the model p value were used to predict model capability.

Table 3.2. Independent variables for the enperimental design.

Independent variable	Variable levels			
	Symbol	Low (-1)	Center (0)	High (+1)
Power of microwave (W)	X_1	100	200	300
Water content in NAES (%, v/v)	X_2	10	30	50
Time (s)	X_3	600	750	900

3.2.4.5.Experimental setup for extraction scale-up

15 g of grape pomace were extracted using 500 mL of ChCit with 30% (v/v) of water in two separate extraction steps. Initially, pomace extraction was performed in 5 minutes with a self-made US Titanium horn prototype equipped with an US generator, and a US converter (20 kHz, power 500 W). Subsequently, grape pomace was extracted in the MW pressurised reactor SynthWave (Milestone, Bergamo - Italy). The extraction was performed under nitrogen pressure (3 bar) to avoid anthocyanin oxidation. The stirrer was set to 100%. A ramp of 1 minute

was set to increase the temperature up to 80°C, and extraction was then set for 9 more min at a power output of 300 W. The recovered extracts were centrifuged and the anthocyanins from supernatant was analysed by HPLC (paragraph 3.2.2.3.).

3.2.4.6.Anthocyanin recovery and NADES recycling

Anthocyanin recovery from the ChCit extracts was achieved via resin adsorption on a glass column (1.1 cm x 11 cm), which was wet-packed with 6 g macroporous resin Sepabeads SP825L (BV= 10.45 mL). Sepabeads SP825L is a nonionic polystyrene-divinylbenzene polymer resin pore size ~1.20 mL g^{-1} pore volume (105 Å mean pore size). Surface area of Sepabeads SP825L is 650 m^2 g^{-1}. Resin was pre-treated with 2 BV of 96% (v/v) ethanol and again with 5 BV of deionised water according to Chen et al. (2015). The extracts prepared with ChCit with 30% (v/v) of water, under optimal conditions on the lab scale, were diluted to 80% (v/v) of water and flowed through the column at flow rate of 1.5 mL min^{-1}. ChCit was eluted from the column with deionised water (3 BV) and the anthocyanins were desorbed using acidified aqueous ethanol (75% (v/v) with 0.1% HCl (v/v)) (3BV). The water fraction was evaporated under vacuum and ChCit was recovered. The water percentage in evaporated ChCit was determined from calibration curve of water percentage in ChCit and refractive index (n_D). The cleanness of the NADES was checked by NMR spectroscopy. ^{1}H NMR spectra from DMSO-d_6 were recorded on a Bruker AV-300 spectrometer, as described in Cvjetko Bubalo et al. (2015b).

Anthocyanins from EtOH fractions were analysed by HPLC, as described in paragraph 3.2.2.3., and yields were calculated.

Experiments for the recovery of anthocyanins and the recycling of NADES on a larger scale were performed on an automatic double column system (‘green technologies development platform’, DSTF, University of Turin, Turin, Italy) by Tecnoimpianti Skid, Italy (Cravotto et al., 2018) in one column (5 cm x 80 cm), equipped with 370 g of Sepabeads SP825L (BV=884 mL), as described above with some changes. The flow rate was 15 mL min^{-1}.

Recycled NADES were diluted with 30% of water and used again for extraction.

3.2.5. Evaluation of biological activities of prepared extracts

3.2.5.2.Oxygen Radical Absorbance Capacity Assay (ORAC)

ORAC was determined according to method described in paragraph 3.2.1.3. with some modification. The final reaction mixture for the assay (3 mL) was prepared as follows: 2.25 mL of 0.04 mL fluorescein sodium salt in 0.075 M sodium phosphate buffer (pH 7.0), and 0.375 mL of 600- 1400 times diluted extracts or 25 mM Trolox as standard. The control was 0.075 M sodium phosphate buffer. The reaction mixtures were incubated for 30 min at 37°C followed by reaction initiation with 0.375 mL 152 mm AAPH and fluorescence was read every minute up to value zero at 485 nm excitation, 520 nm emission. Results were calculated according to eq. 3.4.

3.2.5.3.Determination of antiproliferative activity by cytotoxicity assay

Antiproliferative activity of the grape pomace extracts prepared in NADES and ethanol, as a referent solvent, were evaluated *in vitro* against two adherent human tumour cell lines by the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation (MTS) assay, as described in paragraph 3.2.1.3. HeLa and MCF-7 cells were cultured in DMEM supplemented with 5% FBS and maintained in BioLite petri dishes in the incubator with humidified atmosphere and 5% CO_2 at 37 °C. Individual experiments to test cytotoxicity of the prepared extracts were performed in BioLite 96-wells plates seeded with exponentially growing cells at the concentration (~$3x10^4$ cells per well in 100 µL of media) and incubated for 24 h, after which the treatment was done. Grape pomace extracts (GPCCit, GPEtOH), extraction solvents (ChCit, EtOH) and NADES-forming compounds (Ch, Cit) were diluted in the culture medium when applied to the cells so the final volume ratio was 1%, 5% and 10% (v/v), while control cells were non-treated cells. Upon 72 hours of treatment, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay was preformed according to the manufacturer's instructions with minor modification. Briefly, 10 µL of the MTS reagent was added to each well, and cells were incubated for further 3h, after which absorbance at 490 nm was measured on the microplate reader. Cell viability was expressed as percentage of treated versus control cells. The experiments were performed three times with five parallels for each volume ratio and data were expressed as the means ± S.D.

3.2.5.4.*Evaluation of cell death and cell cycle arrest by flowcytometric analysis*

Quantitative analysis of live, apoptotic and death cells treated with extracts obtained in NADES and ethanol was done by Muse® Cell Analyser using Muse™ Annexin V & Dead Cell

Kit according to the manufacturer's specifications. HeLa cells were seeded into a 6-well culture plates at a density of 5 x 10^4 cells mL^{-1} (2 mL per well) and after overnight growth cells were treated with 5% (v/v) of extracts for 72 h. After treatment, both floating and adherent cells were collected, centrifuged (600 g min^{-1}) and suspended in cell culture medium to adjust the cell concentration according to the manufacturer's protocol. Then, a 100 µL aliquots of cell suspension were added to 100 µL of Muse™ Annexin V & Dead Cell Reagent and incubated in the dark for 20 minutes at room temperature. The cells were then analysed using the Muse® Cell Analyser. Each sample was tested in a duplicate and each experiment was performed twice. The Muse™ Annexin V & Dead Cell Assay detect phosphatidylserine on the external membrane of apoptotic cells by Annexin V-PE binding, while 7-aminoactinomycin D (7-AAD) is used as a dead cell marker. Based on that, this assay detects four distinctive populations of cells: live (Annexin V negative and 7-AAD negative), early apoptotic (Annexin V positive and 7-AAD negative), late stage apoptotic (Annexin V positive and 7-AAD positive) and dead cells, mostly nuclear debris (Annexin V negative and 7-AAD positive).

The Muse™ Cell Cycle Kit coupled with the Muse®Cell Analyzer enables quantitative information on cell cycle distribution. The assay utilizes PI-based staining of DNA content to discriminate and measure the percentage of cells in each phase of cell cycle (G0/G1, S, and G2/M). Analysis was done according to the manufacturer's specifications. HeLa cells were seeded and treated as described for cell death analysis. After 72 hours of treatment, adherent cells were harvested using trypsin-EDTA solution to dissociate the cells from the culture plates and obtain single-cell suspensions. Further step, after adjusting the cell concentration according to the manufacturer's protocol, was fixing of samples by ice-cold 70% ethanol. Such fixed cells are stable for 2 to 3 months at –20°C. At the day of analysis, staining protocol was done, which ends up with suspending of the cell pellet in 200 µL of Muse™ Cell Cycle Reagent and incubation of samples for 30 min at room temperature, protected from light. After that sample was ready for analysis on Muse™ Cell Analyzer.

3.2.5.5.Evaluation of oxidative stress by flowcytometric analysis

The Muse® Oxidative Stress Kit was used in here to measure percentage of ROS (+) cells in order to determine antioxidative potential of extracts toward HeLa cells, in which oxidative stress was induced by H_2O_2. The Muse® Oxidative Stress Reagent is based on dihydroethidium (DHE), which is often used for the detection of reactive oxidative species in cellular populations. HeLa cells were seeded into a 12-well culture plates at a density of 5 x 10^4 cells mL^{-1} (1 mL per well) and after overnight growth pre-treated with 5% (v/v) of extracts for 24 h.

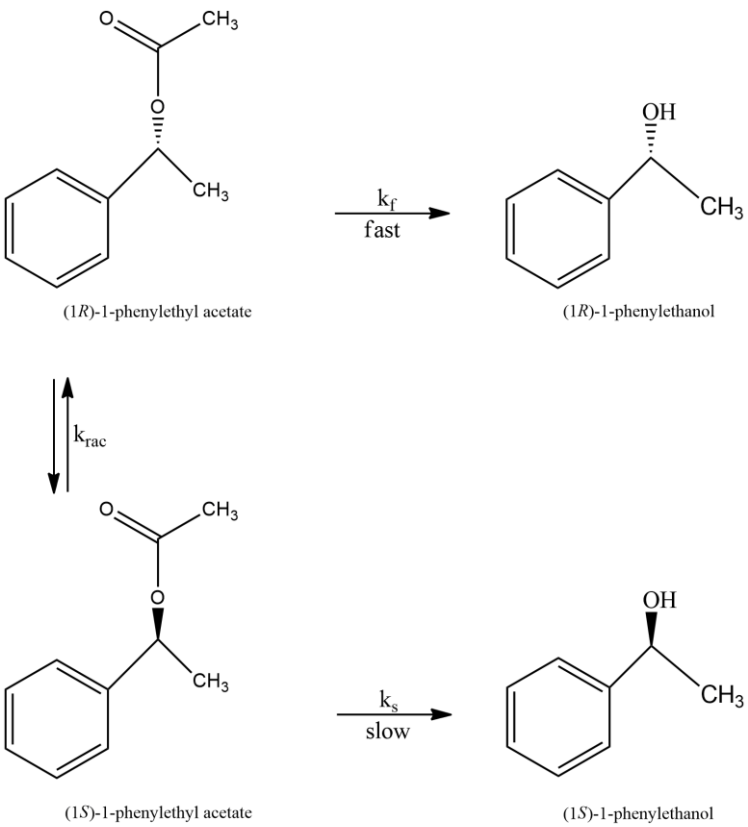
After pre-treatment oxidative stress was induced by 100 μM H_2O_2 for further 4 hours, after which preparation of samples for flowcytometric analysis was done according to the manufacturer's specifications. Briefly, adherent HeLa cells were dissociated from the flask by trypsine-EDTA solution after which cell concentration was adjusted and cell samples were prepared in 1X Assay Buffer before proceeding with staining protocol. Mixture of cells and Muse® Oxidative Stress Reagent was incubated for 30 minutes at 37°C, after which samples were analysed on the Muse® Cell Analyzer.

3.2.6. Methodology for development of environmentally friendly process for the synthesis of (*R*)-1-phenyl ethanol using lipase in NADES

3.2.6.1. NADES screening of lipase catalysed (R)-1-phenylethanol synthesis

For initial screening of NADES the reaction started by adding 5 mg of Novozym 435 to 1 mL of solvent (buffer of NADES) containing 0.05 mol L^{-1} (*R, S*)-1-phenylethyl acetate. Reactions without the enzyme were also performed. For each measurment point separated reaction was set.

The lipase catalysed hydrolysis is represented as:



$$k_{rac} > k_f >> k_s \qquad [3.7.]$$

At specified time intervals, reaction was stopped and 1 mL of water were added, after reaction mixture were extracted with 8 mL of *n*-heptane on vortex shaker (3 min). The organic phase was analysed by gas chromatography.

Determination of (R)-1-phenylethanol concentration

(*R*)-1-phenylethanol, (*S*)-1-phenylethanol, (*R*)-1-phenylethyl acetate, (*S*)-1-phenylethyl acetate were identified by a gas chromatograph with mass spectrometry (GCMS) equipped with a Varian CHIRASIL-DEX CB (25 m x 0,25 mm x 0,25 μm). Helium was used as a carrier gas at a flow rate of 96.9 mL min^{-1}. The injector temperature was (200°C), detector (200°C), while column temperature was 80°C for 2 min, and than temperature was increased from 80°C to 140°C with rate of 5°C min^{-1}. Substrates and products were identified with external standards and the quantification was done with calibration curves of (*R*)-1-phenylethanol (0.409–35 mmol L^{-1}).

The efficiency of proposed biocatalytic reaction was monitored for initial reaction rate, conversion (*X*), enantiomeric excess (*ee*) and volumetric productivity (V_p), (eq. 3.8.-3.11.)

Initial reaction rate (k_o, mmoL L^{-1} min^{-1}) was calculated from the linear parts of the plots of product concentration vs. reaction time according to equation:

$$k_0 = \frac{a_p}{t} \qquad [3.8.]$$

where *a*p is slope of the (mmol L^{-1}), and t is time.

Reaction conversion (*X*, %) was calculated according to equation:

$$X = \frac{c_A}{c_{AT}} \times 100 \qquad [3.9.]$$

where c_A is concentration of (*R*)-1-phenylethanol (mol L^{-1}), and c_{AT} maximal teoretical concentration of (*R*)-1-phenylethanol (mol L^{-1}).

Enantiomeric excess (ee, %) was calculated according to equation:

$$ee = \frac{(R_{OH} - S_{OH})}{(R_{OH} + RS_{OH})} \times 100 \qquad [3.10.]$$

where R_{OH} is area under the curve of (*R*)-1-phenylethanol (mol L^{-1}), and S_{OH} area under the curve of (*S*)-1-phenylethanol (mol L^{-1}).

Productivity (V_P, mmol L^{-1} min^{-1}) was calculated according to equation:

$$V_P = \frac{c_{p2} - c_{p1}}{t} \quad [3.11.]$$

where, c_{P1} is concentration of (*R*)-1-phenylethanol (mol L^{-1}) at 0h, c_{P2} concentration of (*R*)-1-phenylethanol (mol L^{-1}) at the end of the reaction, and *t* is the time of the reaction (min).

All experimental measurements were performed in triplicate.

3.2.6.2. Storage stability of enzyme

The storage stability of Novozym 435 in prepared solvents was measured according to the procedure reported by Cvjetko Bubalo et al. (2015a). Briefly, enzyme was incubated in ChGly, ChGlc and ChEG with 10, 30 and 50% of water, and phosphate buffer on room temperature. At specified time intervals, enzyme reaction was initiated by adding substrate buthyl-acetate and the reaction was monitored through the time on gas chromatograph.

Determination of butyl acetate concentration

Qualitative and quantitative analysis of butyl ethanoate and n-butanol was performed on gas chromatograph. For analyses was used Beta DEX 225 (30 m x 0.25 mm x 0.25 μm) column. Column temperature was T_1 = 55°C (3 min), T_2 =150°C (ΔT = 30°C min^{-1}). Helium was used as a carrier gas at a flow rate 51.0 mL min^{-1}. Substrates and products were identified with external standards and the quantification was done with calibration curves of *n*-butanol (0.025-0.005 mol L^{-1}).

Results were presented as residual enzyme activity.

The residual activity (*A*, %) was calculated according to eq. 3.12.

$$A = \frac{k_1}{k_0} \quad [3.12.]$$

where k_1 is the initial reaction rate obtained by the enzyme after incubation in certain solvent, and k_0 is the initial reaction rate obtained without previous incubation.

Inactivation rate constants (K_d) were calculated according to kinetic of 1st order in StatSoft Statistica 8.0.

3.2.6.3. Optimisation of (R)-1-phenylethanol synthesis in NADES

Optimization of kinetic resolution of (*R*,*S*)-1-phenylethyl acetate in ChGly was performed by Box-Behnken design. Influence of independent variables temperature (X_1, 20–60°C), water content in ChGly (X_2, 10–50%, v/v) and time (X_3, 1-6 h) on depended variables – reaction conversion (%, *X*) were studied (Table 3.3).

Table 3.3. Independent variables for the experimental design

Independent variable	Variable levels			
	Symbol	Low (-1)	Center (0)	High (+1)
time	X_1	0.1	0.55	1
% H2O (v/v)	X_2	10	30	50
T(°C)	X_3	20	40	60

To optimize extraction process, 15 experiments were performed with 3 center points per block, and the response were analysed using the numerical tools provided Design expert (Version 7.0.0., Suite 480, Minneapolis, MN 55413). Each of experiment were performed with 5 mg of Novozym 435, 1 mL of ChGly containing 0.05 mol L^{-1} (*R, S*)-1-phenylethyl acetate The responses were fitted with second-order polynomial equation (RSM model), described in (Sontakke and Yadav, 2011).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad i < j \qquad [3.13.]$$

where X_1, X_2,. . ., X_k are the independent variables and Y depended variable, β_0, β_j (i = 1, 2,. . ., k), β_{ii} (i = 1, 2,. . ., k), and β_{ij} (i = 1, 2,. . ., k; j = 1, 2,. . ., k) are the regression coefficients for the intercept, linear, quadratic and interaction terms, respectively, and k is the number of variables.

To calculate the second-order polynomial coefficients analysis of variance (ANOVA) were applied using the Design-Expert (Version 7.0.0., Suite 480, Minneapolis, MN 55413) software. The value of the determination (R^2) was used to predict model capability.

Through all of 15 experiments, at specified time intervals, reaction was stopped and analysed on GCMS as described in paragraph 3.2.4.1.

3.2.6.4. Downstream process for enzyme and NADES recovery in laboratory scale

After performing kinetic resolution of (*R,S*)-1-phenylethyl acetate at optimal reaction condtions (50.4°C, 47.75% of water in ChGly and 5 h 52 min), the reaction medium with Novozyme 435 was filtered under vacuum. The recovered enzyme was washed with water

(5x10 mL) and ethyl aceate (5x 10 mL) and dried in an oven at 40°C overnight and stored in a fridge until the next run. 5 mg of recovered Novozyme 435 was weight and used again for hydrolysis of (*R,S*)-1-phenylethyl acetate in fresh choline chloride:ethylene glycol with 50% of water. Reaction was monitored and analysed as described in paragraph 3.2.4.1. Recycling and reuse of enzyme is repeated in 5 cycles.

Enzyme reusability (η_{relative} ,%) was calculated according to equaion 3.14.

$$\eta_{relative\,(\%)} = \frac{\eta_{run\,(1+n)}}{\eta_{run\,(1)}} \text{x} 100, n = 0,1,2,3 \ldots. \qquad [3.14.]$$

where, $\eta_{\text{run(1)}}$ is the reaction yield with fresh enzyme, and $\eta_{\text{run(1+n)}}$ is the reaction yield with recycled enzyme, once, twice...,∞.

NADES recycling and recovery was performed as separate experiment. Kinetic resolution of (*R,S*)-1-phenylethyl acetate in optimal condtions were stoped after 5 h and 52 min, enzyme was filtred from reaction medium and a reaction medium was placed in a separatory funnel and extracted with ethyl acetate (5x10 mL). The organic phases were collected and evaporated under reduced pressure, yielding crude products. The regenerated NADES were tested for the four rounds.

NADES reusability (V_{relative}, %) was calculated according to equaion [3.15.]

$$V_{relative\,(\%)} = \frac{V_{run\,(1+n)}}{V_{run\,(n)}} \text{x} 100, n = 1,2 \ldots \qquad [3.15.]$$

where, $V_{\text{run(1)}}$ is the volume of NADES in n-cycle, and $V_{\text{run(1+n)}}$ is the volume of the recycled NADES after n-cycle .

Reaction conversion in recycled NADES were calculated according to eq. 3.9.

(*R*)-1-phenylethanol and unreacted (*S*)-1- phenylethyl acetate from organic fraction were separated on silica gel column (1.2 cm x 5 cm) using *n*-heptane:ethyl acetate (4:1) as mobile phase.

Separation efficiency (η , %) was calculated according to eq. 3.16.

$$\eta = \frac{m_k}{m_0} \qquad [3.16.]$$

where m_k is the mass of the (*R*)-1-phenylethanol after the silica gel column, and m_0 is the theoretical mass of (*R*)-1-phenylethanol or (*S*)-1- phenylethyl acetate before silica gel column.

3.2.6.5. Preparative scale of kinetic resolution of (R,S)-1-phenylethyl acetate in NADES and isolation of (R)-1-phenylethanol

250 mg of Novozyme 435 were added in 500 mL of choline chloride:glycerol with 47.75% of water containing 0.05 mol L^{-1} (*R, S*)-1-phenylethyl acetate. Reaction was performed on 50.4°C for 5h 52 min h on ES-20/60 Orbital Shaker-Incubator. After, Novozyme 435 was filtred from reaction medium under vacuum. The corresponding (*R*)-1-phenylethanol was isolated with extraction using ethyl acetate as the renewable solvent. Extraction was performed in 5 cycles, and ethyl acetate fraction was collected. (*R*)-1-phenylethanol and unreacted (*S*)-1-phenylethyl acetate from organic fraction were separated on silica gel column, as described in paragraph 3.2.4.4.

Reaction yield was calculated according to eq. 3.17.

$$\eta = \frac{m1}{mo} \qquad [3.17.]$$

where m1 is the mass of the (*R*)-1-phenylethanol and (*S*)-1- phenylethyl acetate after reaction, and m_0 is the mass of (*R*)-1- phenylethyl acetate and (*S*)-1- phenylethyl acetate before biocatalytic reaction.

3.2.7. Data analysis

All experimental results were statistically analysed using Statistica 8 software. Data in the text and tables are expressed as the mean ± standard deviation (±SD), and error bars in the figures indicate the SD. The differences between the means were analysed by the ANOVA test, followed by post-hoc Tukey's test. A significant difference was considered at a p value < 0.05.

4. RESULTS

The results of this thesis are presented in three sections: (*i*) development of an environmentally friendly method for isolation of anthocyanins from grape pomace using natural deep eutectic solvents (NADES) and evaluation of biological activities of prepared extracts (*ii*) development of environmentally friendly process for the preparation of (*R*)-1-phenyl ethanol using lipase in NADES.

The first section (4.1.) is focused on experiments related to application of NADES in extraction of anthocyanins from grape pomace with NADES and alternative energy sources. The experimental design in this section entails the following steps: (*i*) NADES selection; (*ii*) extraction method selection and parameter optimisation with scale-up; and (*iii*) the recovery of the target compounds and NADES. Eight NADES have been prepared and fully characterised. The most promising NADES was selected for further optimisation based on physicochemical characteristics, extraction power and extract stability. The influence of microwave and ultrasound, used alone or simultaneously, was also investigated. The parameters that influence the extraction process, such as time, water addition and power of alternative energy sources, microvawe and/or ultrasound, were studied. Finally, the optimised method for the extraction of anthocyanins from grape pomace was performed on a larger scale (0.5 L), with NADES recycling. The experimental design, operational conditions and results are summarised on Figure 4.1.

Focus of this section was also biological activity evaluation of grape pomace-extracts to explore potential for ready-to-use industrial application. To evaluate the biological activity (*i*) antioxidant capacity was determined by ORAC method while the ability of grape pomace extracts to protect cells from induced oxidative was determined *in vitro*, and (*ii*) *in vitro* cytotoxicity of prepared extracts was assessed by antiproliferation assay on two tumour cell lines, whereas for investigation of type of cell death or cell cycle arrest a flow cytometric analysis was applied.

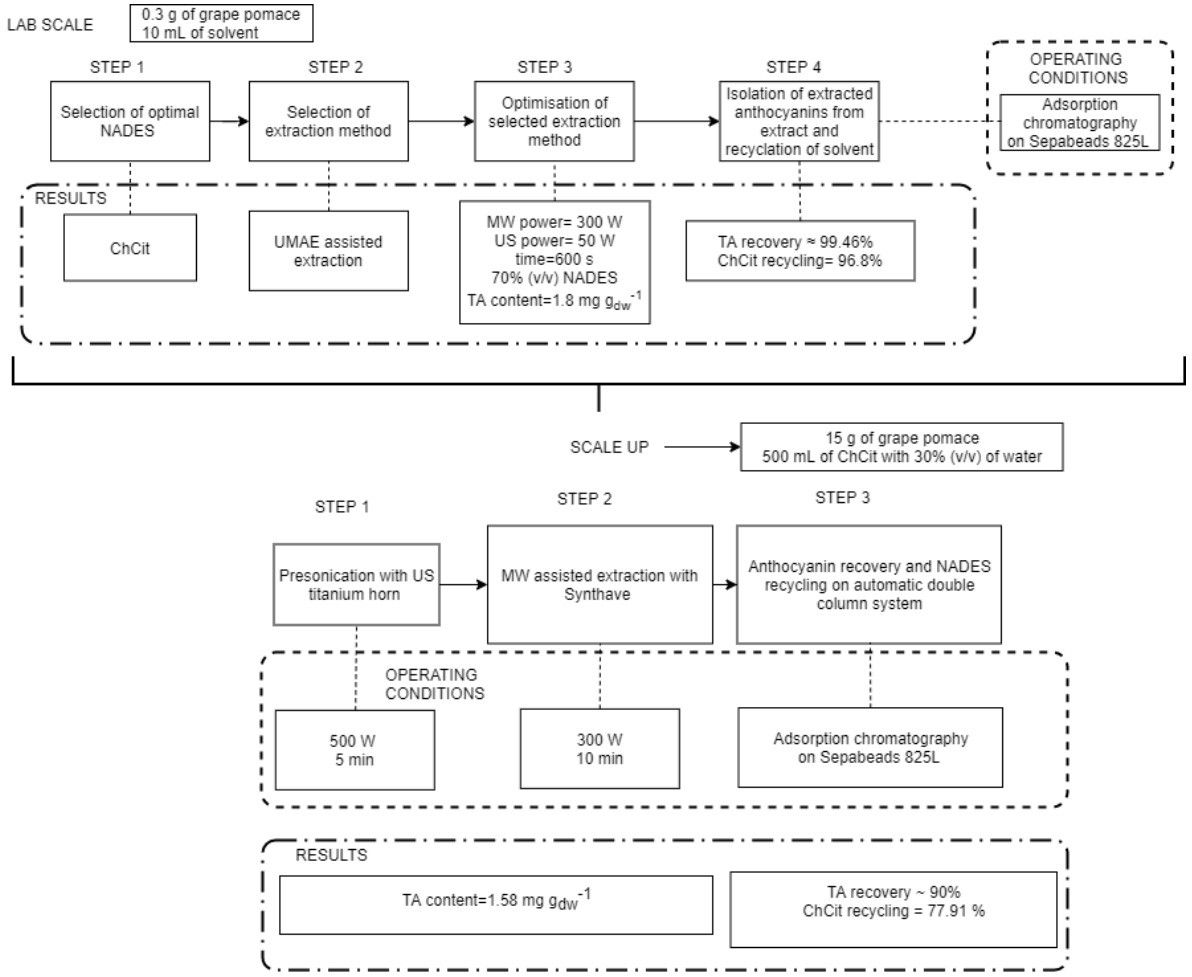


Figure 4.1. Flowchart of experimental design, operational conditions and results of development of an environmentally friendly method for isolating of anthocyanins from grape pomace using NADES.

The second section (4.2.) represents results from application of NADES in lipase-catalysed synthesis of (*R*)-1-phenylethanol (Figure 4.2.). To develop the eco-friendly lipase catalysed process for production of optically pure (*R*)-1-phenylethanol in NADES, the following steps were included: (*i*) selection of NADES for optimal enzyme performance (*ii*) optimization of the biocatalytic process and performing optimal reaction in preparative scale, (*iii*) recovery of the (*R*)-1-phenylethanol/NADES and NADES recycling.

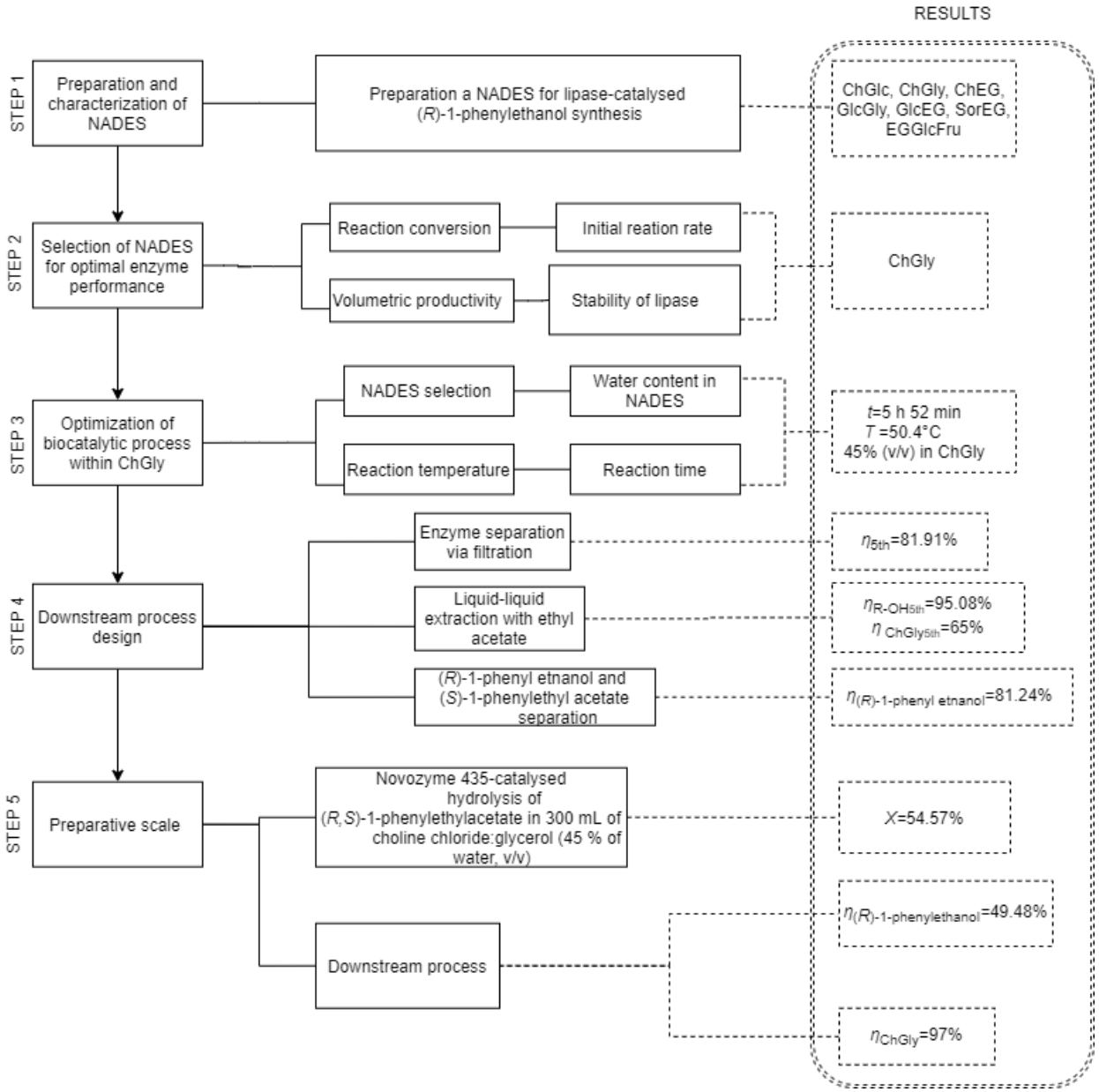


Figure 4.2. Flowchart of experimental design and results of development of an environmentally friendly method for lipase-catalyzed (*R*)-1-phenylethanol production in NADES**.**

4.1. Development of environmentally friendly method for extraction of anthocyanins from grape pomace with NADES

The aim of this part of the research was to develop an eco-friendly extraction method for grape pomace anthocyanins on a larger scale. Results from a preliminary screening of 8 different natural deep eutectic solvents (NADES) were presented on Table 4.1.-4.2 and Figures 4.3.-4.4. The effects of microwave (MW), and ultrasound (US) irradiation (used alone or simultaneously), as well as that of process parameters on extraction efficiency have been investigated in order to maximise anthocyanin extraction yield. Those results were presented on Figures 4.5.-4.6. and Tables 4.3.-4.4. The optimised procedure was scaled up to a half- litre batch (paragraph 4.1.2.5.). Anthocyanins were recovered from NADES, which were recycled (Figure 4.7.)

4.1.1. NADES selection

To select the optimal NADES, properties, extraction efficiency and anthocyanins stability in prepared extracts were considered. Physicochemical properties and cytotoxicity of tested NADES are presented in Table 4.1. Extraction efficiency with different NADES are presented on Figure 4.1. Anthocyanins from prepared extracts were identified and quantified with high performance liquid chromatography (HPLC) (Table 4.2.). The storage stability of anthocyanins in the prepared extracts with NADES and conventional solvents are presented on Figure 4.3.

Table 4.1. Used NADES (25%, v/v) and their properties.

NADES[b]	Abbreviation	Price (€ kg^{-1})[a]	pH	Polarity [E_{NR}kcal mol^{-1}]	Viscosity [Pas]	ORAC [μmol TE g^{-1}]
Choline chloride: citric acid	ChCit	46.79	0.93	49.39	0.0169	1.7499
Choline chloride: malic acid	ChMa	52.72	0.67	49.93	0.0179	11.9194
Choline chloride: proline: malic acid	ChProMa	252.42	3.21	50.50	0.0121	2.6140
Proline: malic acid	ProMa	351.18	2.67	50.19	0.0150	7.6378
Betaine: malic acid	BMa	81.23	3.27	50.50	0.0462	7.2669
Betaine: citric acid	BCit	63.79	2.60	50.01	0.0554	1.6130
Malic acid:glucose:glycerol	MaGlcGly	15.02	1.15	50.63	0.0447	4.5132
Malic acid:glucose	MaGlc	17.90	0.49	49.67	0.0817	2.5435

[a] The price of solvents was estimated based od price of raw materials according to the website of Merck (Germany)

[b] EC_{50} for all solvents determined on human cell lines (HEK293T, HeLa, MCF-7) was > 2000 mg L^{-1} i.e. they are non-cytotoxic

All solvents prepared were polar, with polarities similar to water, and pH values were from 0.49 to 3.27. Viscosities od NADES were in range from 0.0169-0.0817 Pas. The estimated cost of tested mixtures ranged from 15.02 € kg^{-1} (MaGlcGly) to 351.18 € kg^{-1} (ChProMa). The ORAC values of the prepared NADES were between 1.67 and 1.87 μmol TE g_{dw}^{-1}, with the highest antioxidant activity obtained for the ChMa, followed by ProMa ≈ BMa > MaGlcGyl > ChProMa ≈ MaGlc >BCit ≈ ChCit. The results demonstrate that the tested NADES had no inhibiting effect on growth of human HEK293T, HeLa, and MCF-7 cells (Table 4.1).

Further, grape pomace extracts were prepared with characterised NADES. Content of extrated total anthocyanins with different NADES was present on Figure 4.3.

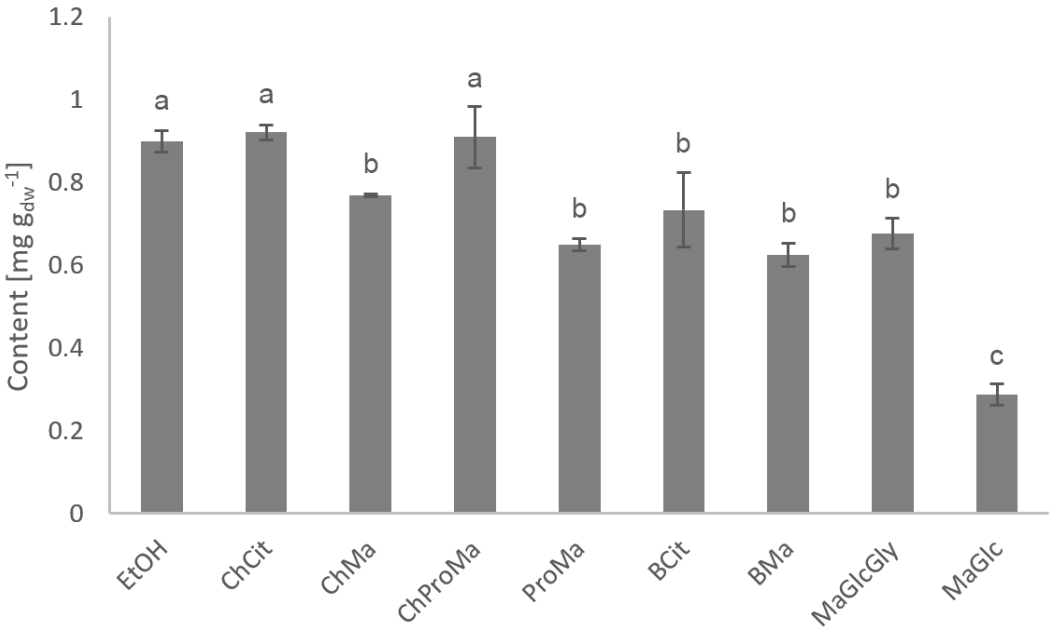


Figure 4.3. Total anthocyanins content (mg g^{-1} dw of pomace) of extract prepared by different NADES. Total anthocyanins were expressed as a sum of identified anthocyanins by HPLC. Content of anthocyanins were expressed as the means (n=3) ± S.D. Presented value followed by different lower-case letters (a-c) are significantly different (p<0.05) as measured by Tukey's HSD test.

The highest content of total anthocyanins (sum of identified anthocyanins by HPLC) was extracted with ChCit ≈ ChProMa ≈ EtOH, followed by ChMa ≈ BCit ≈ MaGlcGly ≈ ProMa ≈ BMa > MaGlc. Furthermore, values ranged from 0.28 to 0.92 mg g_{dw}^{-1} (Figure 4.3.). Content of identified anthocyanins was present in Table 4.2.

Table 4.2. Anthocyanins content (mg kg^{-1} dw of grape pomace) of prepared extract with different NADES. Content of anthocyanins were expressed as the means (n=3) ± S.D.

Prepared extracts	Dp	Pt	Pn	Mv	PnAc	MvAc	CyCm	PnCm	MvCm
EtOH	22.1±3.30	32.2±4.18	21.5±2.69	226.7±10.14	37.9±0.61	33.1±1.73	53.5±4.96	40.6±1.02	444.9±2.30
ChCit	32.8±0.34	48.5±0.01	22.8±0.43	285.6±5.45	37.5±0.54	34.3±1.67	45.5±0.93	36.8±0.97	387.0±7.52
ChMa	26.2±0.81	40.3±1.27	18.2±0.58	239.1±2.66	30.0±0.25	29.6±0.80	36.0±1.06	29.4±0.07	317.5±6.94
ChProMa	50.03±0.00	49.2±31.82	25.5±2.48	285.0±24.75	28.8±2.48	27.8±2.48	44.8±2.48	34.6±2.48	403.3±2.48
ProMa	31.02±0.00	35.9±1.77	9.6±1.77	221.5±1.77	6.8±1.77	23.9±1.77	26.2±1.77	17.8±1.77	285.3±1.77
BCit	21.6±1.23	32.3±16.54	15.7±9.00	180.9±93.16	24.5±12.55	20.7±10.63	29.0±14.21	23.6±12.05	235.8±19.13
BMa	27.1±0.06	38.9±1.54	20.8±1.21	190.2±1.99	30.8±0.66	26.3±0.30	35.7±2.09	29.4±0.40	239.5±22.15
MaGlcGly	25.5±1.18	37.8±1.99	17.1±0.17	214.0±8.51	26.5±1.50	22.9±0.64	31.5±2.14	24.9±1.39	256.8±20.52
MaGlc	10.4±1.16	15.5±1.71	6.7±0.70	88.2±8.93	11.0+1.00	9.3±0.89	13.1±1.21	10.2±1.00	109.3±9.52

*Dp, delphinidin-3-*O*-monoglucoside; Pt, petunidin-3-*O*-monoglucoside; Pn, peonidin-3-*O*-monoglucoside; Mv, malvidin-3-*O*-monoglucoside; PnAc, peonidin-3-*O*-acetylmonoglucoside; MvAc malvidin-3- acetylmonoglucosides; CyCm, cyanidin-3-(6-*O*-p- coumaroyl)monoglucoside; PnCm, peonidin -3-(6-*O*-p-coumaroyl)monoglucosides; MvCm, malvidin-3-(6-*O*-p-coumaroyl)monoglucosides

Four anthocyanin-3-*O*-monoglucosides (delphinidin, petunidin, peonidin, malvidin), two acylated derivatives (malvidin- and peonidin-3- acetylmonoglucosides) and two coumaroyl derivatives (peonidin- and malvidin-3-(6-*O*-p-coumaroyl) monoglucosides) were observed, with malvidin-3-*O*-monoglucoside being most abundant (Table 4.2).

As the extraction efficiency was not significantly different ($p < 0.005$) for ChCit, ChProMa and acidified aqueous ethanol, the stability of anthocyanins in those solvents at −18°C, 4°C and 25°C for 60 days was also considered prior to NADES selection (Figure 4.4.)

Generally, no differences in anthocyanin stability in the various solvents were observed at room temperature, while ChCit was found to possess the highest stabilising capacity at 4°C and −18°C. At 4°C, 70% of anthocyanins were degraded in ChProMa, while in ChCit it was only 14% where as in EtOH was 50% of anthocyanins (Figure 4.4.).

An analysis of all observed criteria (physicochemical characteristics, extraction efficiency, stability of anthocyanins and solvent cost) led to selection of ChCit as the best solvent for the isolation of grape-pomace anthocyanins, and it was thus used in further experiments.

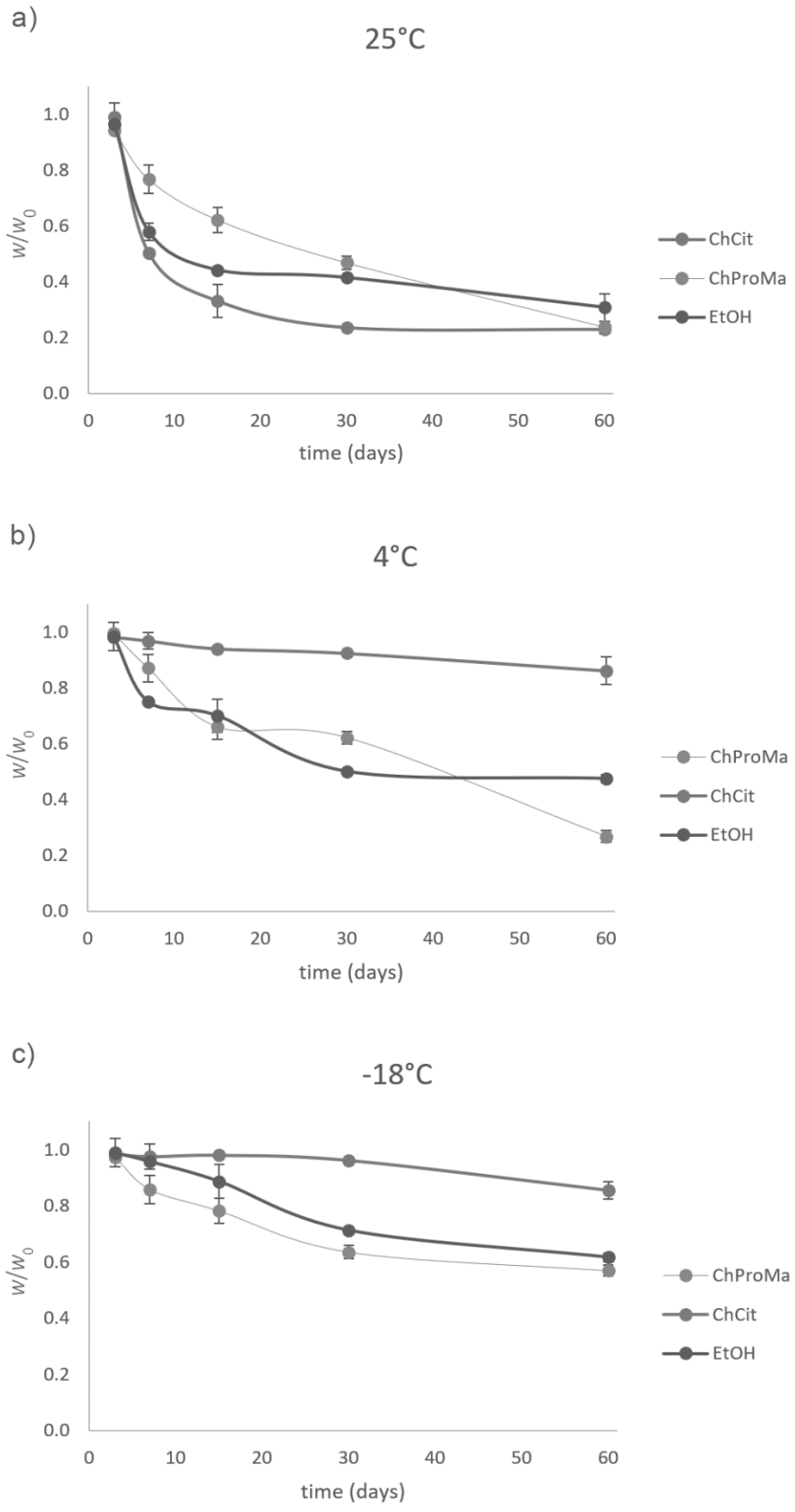

a)
25°C
1.0
0.8
0.6
0.4
0.2
0.0
w/w0
0
10
20
30
40
50
60
time (days)
ChCit
ChProMa
EtOH
b)
4°C
1.0
0.8
0.6
0.4
0.2
0.0
w/w0
0
10
20
30
40
50
60
time (days)
ChProMa
ChCit
EtOH
c)
-18°C
1.0
0.8
0.6
0.4
0.2
0.0
w/w0
0
10
20
30
40
50
60
time (days)
ChProMa
ChCit
EtOH

Figure 4.4. Degradation curve of anthocyanins in ChProMa- ChCit- and EtOH- extracts at 25°C (a), 4°C (b) and -18°C (c) through 60 days. Results were expressed as the means (n=3) ± S.D

4.1.2. Extraction technology selection, extraction-parameter optimisation and scale-up

After selection of optimal solvent, extraction method was selected. Innovative technologies (US, MW, and UMAE) were considered as a means to reduce energy consumption and increase extraction efficiency (Figure 4.5.).

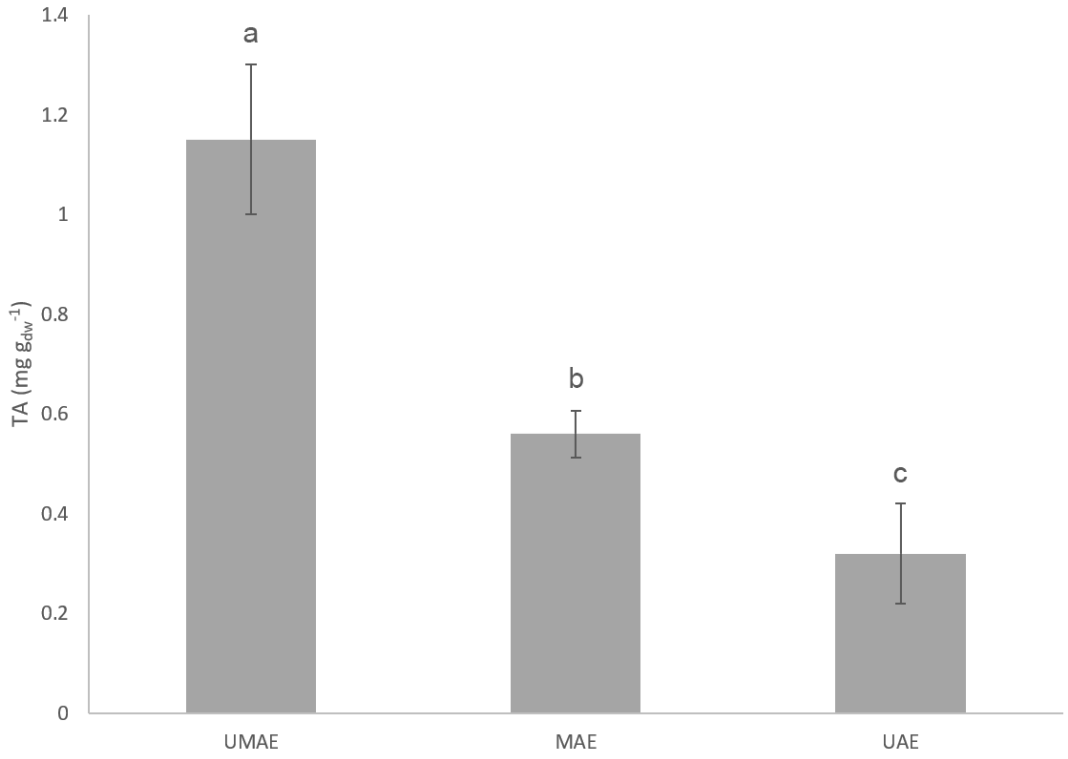


Figure 4.5. Extraction efficiency for grape pomace anthocyanins with ChCit under different irradiation – UMAE- simultaneous ultrasound/microwave-assisted extraction; MAE- microwave-assisted extraction; UAE- ultrasound-assisted extraction. Content of anthocyanins were expressed as the means (n=3) ± S.D. Presented value followed by different lower-case letters (a-c) are significantly different ($p < 0.05$) as measured by Tukey's HSD test.

Anthocyanin contents were extracted in the following order, highest to lowest, UMAE > MAE > UAE (Figure 4.5.). UMAE irradiation was therefore selected as the extraction method.

4.1.2.1. Laboratory process parameters optimisation of the microwave-ultrasound-assisted extraction process

Respone surface methodology was applied to optimise the independed variables - extraction time, power of MW irradiation and water content in the choline chloride:citric acid (ChCit) to have the highest impact on extraction efficiency. These three independent variables were coded at one level, which resulted in the experimental design given in Table 4.3.

Table 4.3. Experimental matrix and values of observed response.

Number of experiment	Variable X_1	Variable X_2	Variable X_3	Response
	P [W]	*t* [s]	*%NADES* [%, w/w]	*w [TA]* [mg g_{dw}^{-1}]
1	100	600	70	0.96
2	100	900	70	1.41
3	300	600	70	1.8
4	300	900	70	1.53
5	200	600	50	1.14
6	200	900	50	0.95
7	200	600	90	0.1
8	200	900	90	0.63
9	100	750	50	0.67
10	300	750	50	1.14
11	100	750	90	0.05
12	300	750	90	0.5
13	200	750	70	1.15
14	200	750	70	1.09
15	200	750	70	1.19

* power of MW – *P*, time – *t*, content of ChCit - *%NADES, w [TA]*- Total anthocyanin content

The content of total anthocyanins ranged from 0.1 to 1.8 mg g^{-1} of dw.

Furthermore, the RSM model was evaluated and ANOVA was used to calculate the statistical significance of the quadratic model, as well as the regression coefficients of the model for each response, and the results are summarised in Table 4.4.

Table 4.4. Analysis of variance (ANOVA) of the modelled responses.

Source	*Sum of squares*	*Degree of freedom*	*Mean square*	*F-value*	*p-value*
Model	3.39	9	0.38	135.46	<0.0001
X_1	0.064	1	0.064	23.05	0.0049
X_2	0.54	1	0.54	195.19	<0.0001
X_3	0.85	1	0.85	306.56	<0.0001
$X_1 X_2$	0.21	1	0.21	76.76	0.0003
$X_1 X_3$	0.13	1	0.13	46.68	0.001
$X_2 X_3$	0.0001	1	0.0001	0.036	0.8571
X_1^2	0.11	1	0.11	39.67	0.0015
X_2^2	0.012	1	0.012	4.48	0.088
X_3^2	1.38	1	1.38	495.55	<0.0001
Residual	0.014	5	0.00278		
Lack of fit	0.00886	3	0.00295	1.17	0.4919
Pure error	0.00505	2	0.00253		
Total	3.41	14			
R^2	0.9960				

$p < 0.01$ highly significant; $0.01 \leq p < 0.05$ significant; $p \geq 0.05$ not significan
X_1: microwave power; X_2: time; X_3: ChCit concentration

The determination coefficient (R^2) of 0.9960 indicates that the quadratic regression model was a good fit for the extraction of grape pomace anthocyanins with NADES in UMAE conditions. The F-value (135.46), with a very low *p*-value (< 0.0001), of the model indicated that the quadratic model was statistically significant (Table 4.4.).

According to the results of the multiple regression analysis presented in Table 4.4. the relationship between the independent variables and the response can be expressed, in coded values, as:

$$Y_1 = 1{,}14 + 0{,}09 * X_1 + 0{,}26 * X_2 - 0{,}33 * X_3 - 0{,}23 * X_1 * X_2 + 0{,}18 * X_1 * X_3 - 0{,}005 * X_2 * X_3 + 0{,}18 * {X_1}^2 + 0{,}058 * {X_2}^2 - 0{,}61 * {X_3}^2 \quad [4.1]$$

where, Y_1 is the content of grape pomace anthocyanins, X_1 is MW power, X_2 is time of extraction and X_3 is the water content in NADES.

The experimental data acquired were used for the creation of three-dimensional response–surface plots for each of the responses (Figure 4.6.).

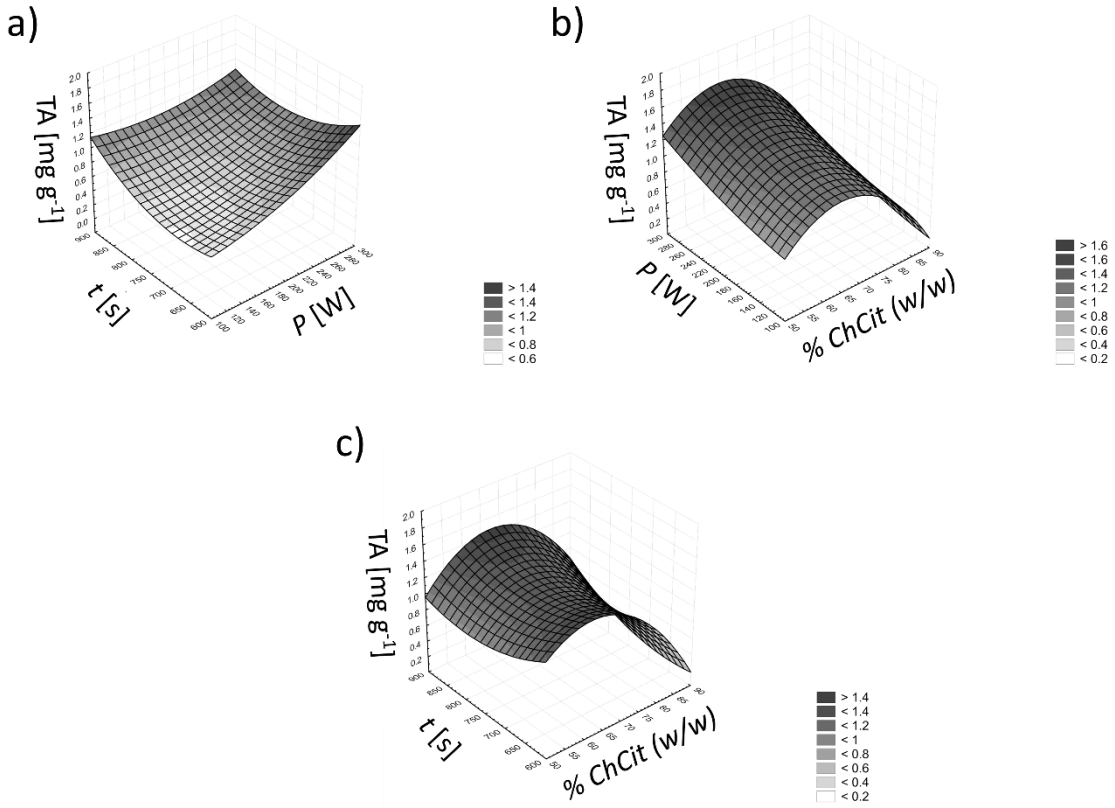


Figure 4.6. Response surface plots showing combined effects of microwave power (*P*) and time (*t*) (a), microwave power and ChCit content (b) or ChCit content and time (c) on content of total anthocyanins (TA).

The optimal values for the maximum response obtained using the Box-Behnken method was 1.8 mg g^{-1}_{dw}. The independent variables were: X_1= 300 W, X_2=600 s and X_3=30% of water in NADES (%).

The verification and validity of the generated mathematical model were performed via the extraction of anthocyanins at these optimal conditions. After performing that experiment under the optimal conditions, extraction efficiency was 1.77 mg g^{-1}_{dw}.

4.1.2.3. Scale-up of laboratory optimised method of extraction

Extraction was performed on a larger scale over 2 separate steps with 15 g of pomace and 500 mL of ChCit; US pretreatment, in the form of the sonication of grape pomace in ChCit (500 W, 5 min), followed by MW-assisted extraction (300 W, 10 min, 30% of water in NADES). Under such conditions, 1.58 ± 0.12 mg g_{dw}^{-1} of anthocyanins were extracted, which is equal to laboratory-scale results.

4.1.3. Recovery of anthocyanins and NADES recycling

Using the adsorption chromatography, anthocyanins were isolated from prepared extract at optimal conditions. As stationary phase, macroporous resin Sepabeads 825L was used, on which anthocyanins from extract were adsorbed. NADES was eluted with deionized water, and anthocyanins were desorbed from resin with acidified aqueous ethanol (70% (v/v) with 0.1% (v/v) of HCl). From the fraction with NADES, water was evaporated. Finally, recycled NADES was reused for extraction of anthocyanins from grape pomace. The recycling yield of ChCit in this system was 96.8%, and anthocyanin recovery was 99.46% (data not shown). The cleanness of the NADES after recycling was checked using NMR spectrometry. The ^{1}H NMR spectra of freshly synthesised and recycled NADES were recorded for that purpose (Figure 4.7.).

Anthocyanins from an extract that was prepared on a larger scale were recovered and the NADES was recycled on an automatic double-column system. The recycling yield of ChCit in this system was 77.91%, and anthocyanin recovery was ~90%.

The regenerated solvent was then used for the extraction of grape pomace anthocyanins. Extraction efficiency with the recycled solvent was 1.42±0.11 mg g^{-1}.

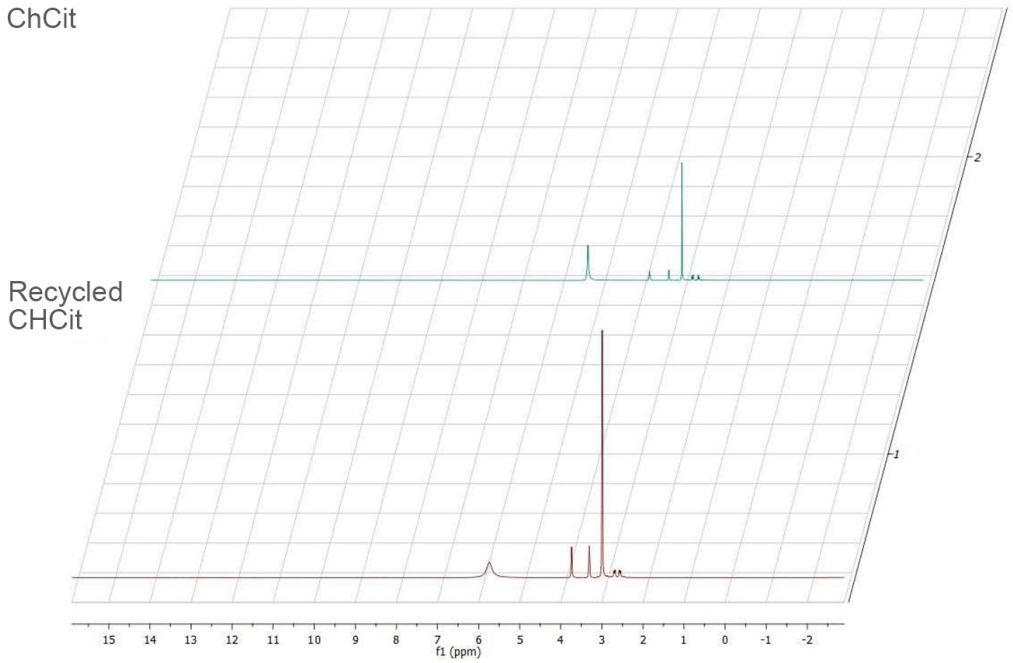


Figure 4.7. ^{1}H NMR spectra of fresh and regenerated NADES ChCit.

4.1.4. Biological activity evaluation of grape pomace-extracts prepared in optimal conditions

The aim of this part of the research was to evaluate potential biologically activity of polyphenolic extracts prepared from grape pomace with ChCit (30% of water, v/v). Biological activities of extracts prepared in NADES were valorised and compared to extracts obtained by ethanol, as a referent conventional solvent. Determination of biological activities included measurement of the antioxidant capacity by ORAC (Table 4.5.), evaluation of oxidative stress by flow cytometric analysis (Figure 4.11.) *in vitro* analysis of cell populations undergoing oxidative stress and *in vitro* cytotoxicity on two tumour cell lines (HeLa and MCF-7) (Figure 4.8.). Also, to investigate possible mechanism of action related with observed cytotoxicity of prepared extracts on HeLa cells, a flow cytometric analysis was applied to determine type of cell death and cell cycle arrest (Figure 4.9. & 4.10.).

4.1.4.1. Chemical characterization of grape pomace extracts

The polyphenolic profile was determined. Also, antioxidant activity of the prepared extracts was determined by the ORAC method and results were presented in Table 4.5.

Table 4.5. Polyphenolic content (mg kg^{-1} dw of pomace), and ORAC value (μmol TE g^{-1} dw of grape pomace) of prepared extract. Content of compounds and ORAC value were expressed as the means (n=3) ± S.D.

	Extracts*	
Compound	GPEtOH	GPChCit
gallic acid	nd**	94.05 ± 6.06
catehin	266.61 ± 6.28	178.90 ± 17.21
quercetin-3-*o*-glucoside	67.00±5.26	94.33±11.15
delphinidin-3-*o*-monoglucoside	41.38±6.60	66.29±0.68
petunidin-3-*o*-monoglucoside	61.02±8.36	98.46±0.02
peonidin-3-*o*-monoglucoside	46.47±5.38	45.77±0.85
malvidin-3-*o*-monoglucoside	412.44±20.28	573.83±10.90
peonidin-3-acetylmonoglucosides	76.23±1.21	75.59±1.08
malvidin-3-acetylmonoglucosides	65.27±3.4	67.80±3.34
cyanidin-3-(6-*o*-p-coumaroyl)monoglucosides	103.38±9.92	91.30±1.86
peonidin -3-(6-*o*-p-coumaroyl)monoglucosides	81.40±2.04	73.72±1.93
malvidin-3-(6-*o*-p-coumaroyl)monoglucosides	826.36±4.60	777.44±15.04
total polyphenols**	2647.48 ± 53.33[b]	2892.07 ± 60.12[a]
ORAC**	1229.65 ± 56.00[b]	2189.97 ± 15.07[a]

* GPEtOH- grape pomace extract in aqueous ethanol (70%, v/v); GPChCit- grape pomace extract in ChCit (70%, v/v)

**not detected

***presented value followed by different lower-case letters (a-b) are significantly different ($p < 0.05$) as measured by Tukey's HSD test.

Considering solvent extractability, higher content of polyphenols from pomace was obtained with NADES ChCit. In grape pomaces extracts prepared with EtOH and ChCit several polyphenolic compounds were identified and quantified including anthocyanins, gallic acid, (+)-catechin and quercetin-3-*O*-glucoside, with the highest content of anthocyanins.

The ORAC values of the extracts obtained was 1229.65 for GPEtOH in comparison to 2189.97 mmol TE g^{-1} dw of pomace for GPChCit.

4.1.4.2. Antiproliferative activity of grape pomace extracts on HeLa and MCF-7 cell lines

Biological evaluation of grape pomace polyphenolic extracts, was conducted on two human tumour cell lines. Cytotoxicity of extracts (GPChCit, GPEtOH), extraction solvents (ChCit and EtOH) and NADES-forming compounds (ChCl and Cit) was evaluated by CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay (MTS test) on HeLa and MCF-7 cells (Figure 4.8.).

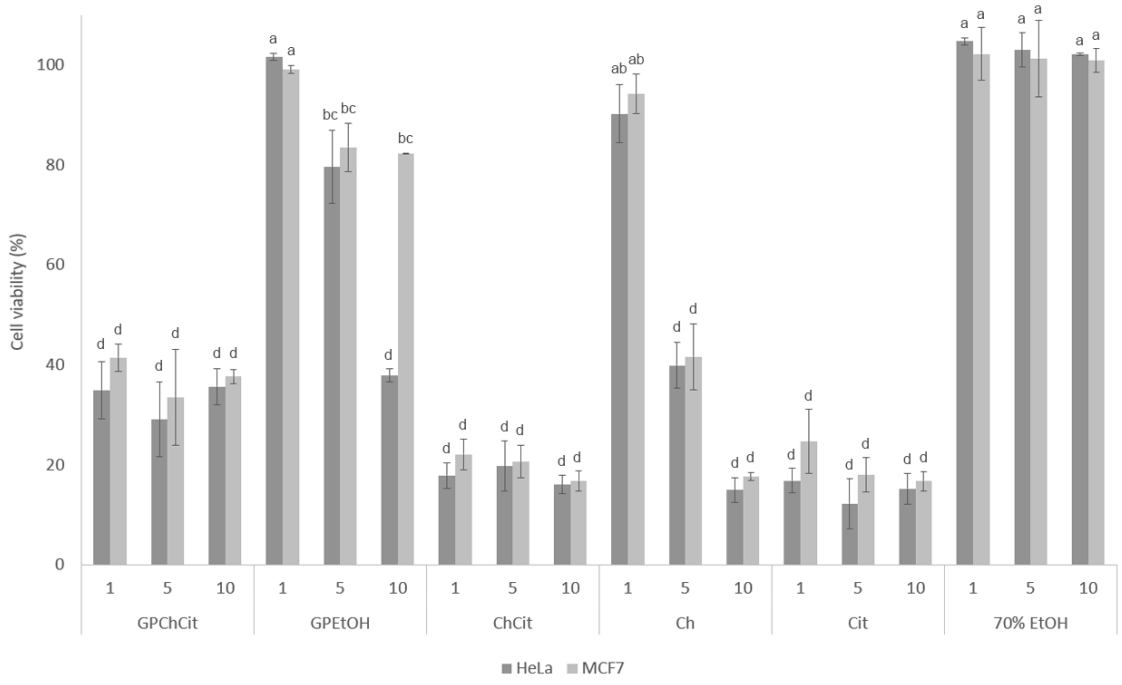


Figure 4.8. Effect of prepared extracts, solvents and NADES-forming compounds on HeLa and MCF-7 cell viability determined by the MTS assay was assessed in volume ratio 1%, 5% and 10% (v/v). Cell viability (%) was expressed as percentage of treated cells versus control cells and the data were expressed as the means (n=5) ± S.D.

Extracts obtained by ethanol (GPEtOH) have not had strong impact on cell viability of HeLa and MCF-7 cells, while rather low cell viability (from 12.19% to 37.61%) was observed in both cell lines treated with GPChCit, ChCit and Cit.

4.1.4.3. Evaluation of cell dead and cell cycle on HeLa cells

Since significant antiproliferative effect of GPChCit was observed, it was interesting to elucidate whether inhibition of cell growth was related to cell cycle arrest or cell death and therefore flow cytometry by Muse® Cell Analyzer and related assays was employed to obtain quantitative data at the single cell level (Figure 4.9. & 4.10.).

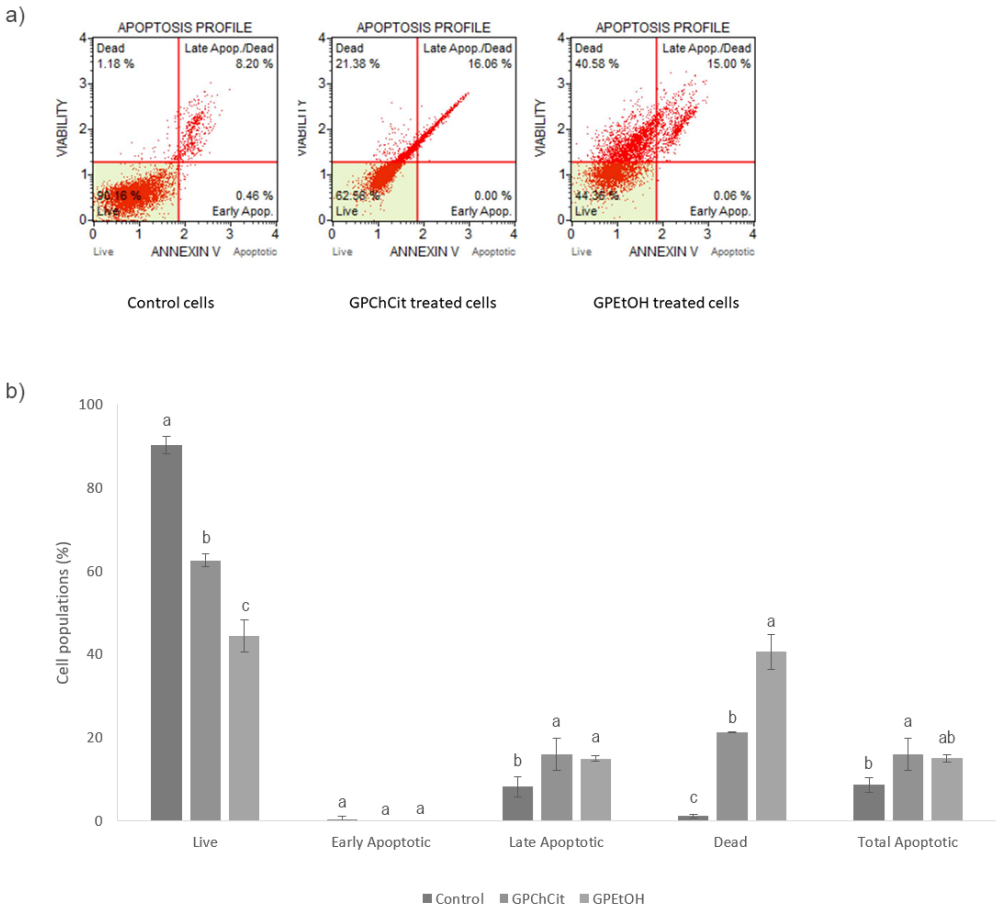


Figure 4.9. Representative histograms of the muse Annexin V & Dead Cell assay are showed in (a) for control cells, GPChCit- treated cells, and GPEtOH-treated cells, while distribution of cell populations is showed in (b). Distribution of cell populations (%) were expressed as the means (n = 3) ± S.D. Non-identical lower-case letters in every group of results (a–c) indicate signifcant difference ($p< 0.05$) determined by Tukey's HSD test.

According to the results presented on Figure 4.9. observed inhibition of cell growth by GPChCit could not be primary related to induction of apoptosis in HeLa cells, since there is no

notable increase in number of apoptotic cells (early, late, total) when compared to control, untreated cells.

Further, evaluation of cell cycle on HeLa cells was conducted (Figure 4.10.)

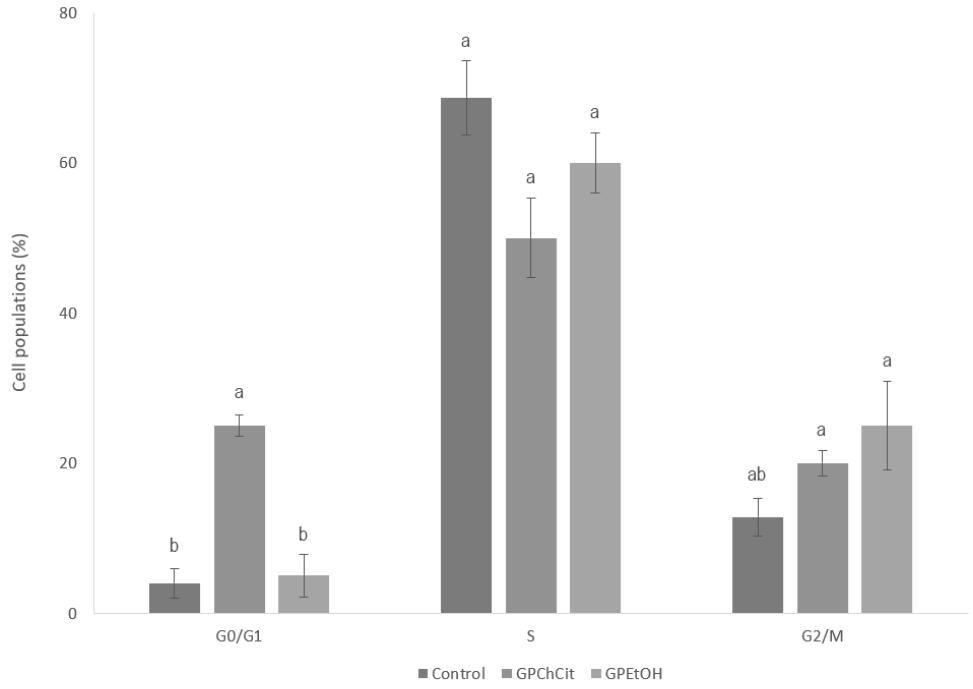


Figure 4.10. Cell cycle analysis of cells GPChCit- treated cells, and GPEtOH-treated cells, while distribution of cell populations is showed in (b). Distribution of cell populations (%) were expressed as the means (n = 3) ± S.D. Non-identical lower-case letters in every group of results (a–b) indicate signifcant difference ($P < 0.05$) determined by Tukey's HSD test.

As it could be seen at Figure 4.10. treatment of HeLa cells with extract prepared with ChCit significantly affected the distribution of cells in G0/G1 phase and reduced number of cells in S phase when compared to control cells. A slightly increas in G2/M population of cells, statistically significant when compared to control, was observed in the case of GPChCit and GPEtOH.

4.1.4.4. Evaluation of oxidative stress by flowcytometer analysis

Furthermore, since it was determined by ORAC method that grape pomace extracts possess antioxidative capacity, their ability to protect cells from induced oxidative stress were tested on HeLa cells. Cell viability was determined and relative percentage of ROS positive cells was measured at the Muse® Cell Analyzer (Figure 4.11.).

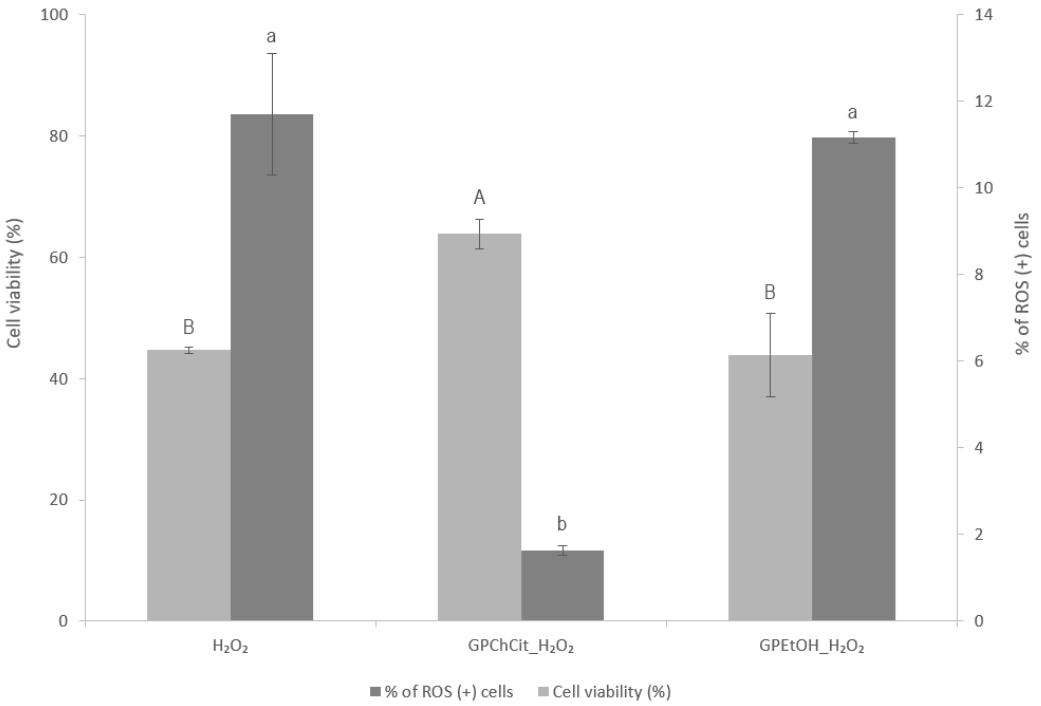


Figure 4.11. Cell viability (%) of HeLa cells measured by MTS assay and percentage of ROS (+) cells pre-treated with 5% (v/v) grape pomace extracts in NADES and conventional solvent. Oxidative stress was induced with H_2O_2 (100 μM) after 24 h pre-treatment. Cell viability was expressed as percentage of treated cells vs. control cells and the data were expressed as the means (n = 5) ± S.D., as well as % of ROS (+) cells. Each sample followed by nonidentical letters (a-b and A-B for cell viability (%) and % of ROS (+) cells respectively) indicate signifcant difference ($p < 0.05$) determined by Tukey's HSD test.

Results presented at Figure 4.11. showed statistically significant increase in HeLa cell viability when pretreatment was done by GPChCit (63.88%) compared with H_2O_2 alone (44.87%) as well as decrease in % of ROS (+) cells from 11.7 % in H_2O_2 treated HeLa cells to 1.63 % in GPChCit-treated cells.

4.3. Development of environmentally friendly process for the synthesis of (*R*)-1-phenyl ethanol using lipase in NADES

The aim of part of the research was to produce optically pure (*R*)-1-phenylethanol, an important building block in drug, agrochemicals and fine chemical industries. For that purpose, lipase-catalyzed kinetic resolution of (*R,S*)-1-phenylethyl acetate within NADES was performed. When scheming this research, further points were included in designing a biocatalytic involving NADES: (*i*) selection of NADES for optimal enzyme performance

(Table 4.6., Figure 4.12-4.13.); (*ii*) optimization of the biocatalytic protocol with performing reaction under optimum condition on half liter batch (Table 4.15.-4.17; Figure 4.14.); (*iii*) recovery of the (*R*)-1-phenylethanol/ChGly (Figure 4.15. & Table 4.18.).

4.3.1. Screening of NADES for optimal lipase-catalysed (*R*)-1-phenylethanol synthesis

To select the optimal NADES for production of (*R*)-1-phenylethanol with hydrolysis of (*R, S*)-1-phenylethyl acetate, prepared NADES were characterized (Table 4.6.), and screened for the optimal enzyme activity (initial reaction rate, volumetric productivity and conversion) (Figure 4.12.). Enantioselective hydrolysis was also performed in 0.025 M potassium phosphate buffer as a reference solvent.

Table 4.6. Used NADES (50%, v/v) and their properties.

NADES[b]	Abbreviation	Price[a] [€ kg^{-1}]	Polarity [kcal mol^{-1}]	pH
Choline chloride:glucose	ChGlc	27.95	50.10	4.03
Choline chloride:glycerol	ChGly	35.93	50.10	3.35
Choline chloride:ethylene glycol	ChEG	32.85	50.28	5.93
Glucose:glycerol	GlcGly	5.57	50.37	6.25
Glucose:ethylene glycol	GlcEG	7.89	50.37	5.21
Sorbose:ethylene glycol	SorEG	656.89	50.37	4.80
Ethylene glycol:glucose:fructose	EGGlcFru	7.42	50.19	4.41

[a] The price of solvents was estimated based od price of raw materials according to the website of Merck (Germany)

[b] EC_{50} for all solvents determined on human cell lines (HEK293T, HeLa, MCF-7) was > 2000 mg L^{-1} i.e. they are non-cytotoxic

As for price of NADES, estimated price based on cost of raw materials varies from approx 5.57 € kg^{-1} for GlcGly up to 656.89 € kg^{-1} for SorEG. NADES selected were weakly acidic to neutral (pH 3.35–6.25) (Table 4.6.).

Further, Novozyme 435-catalysed (*R*)-1-phenylethanol synthesis with prepared and characterised NADES was performed (Figure 4.12.).

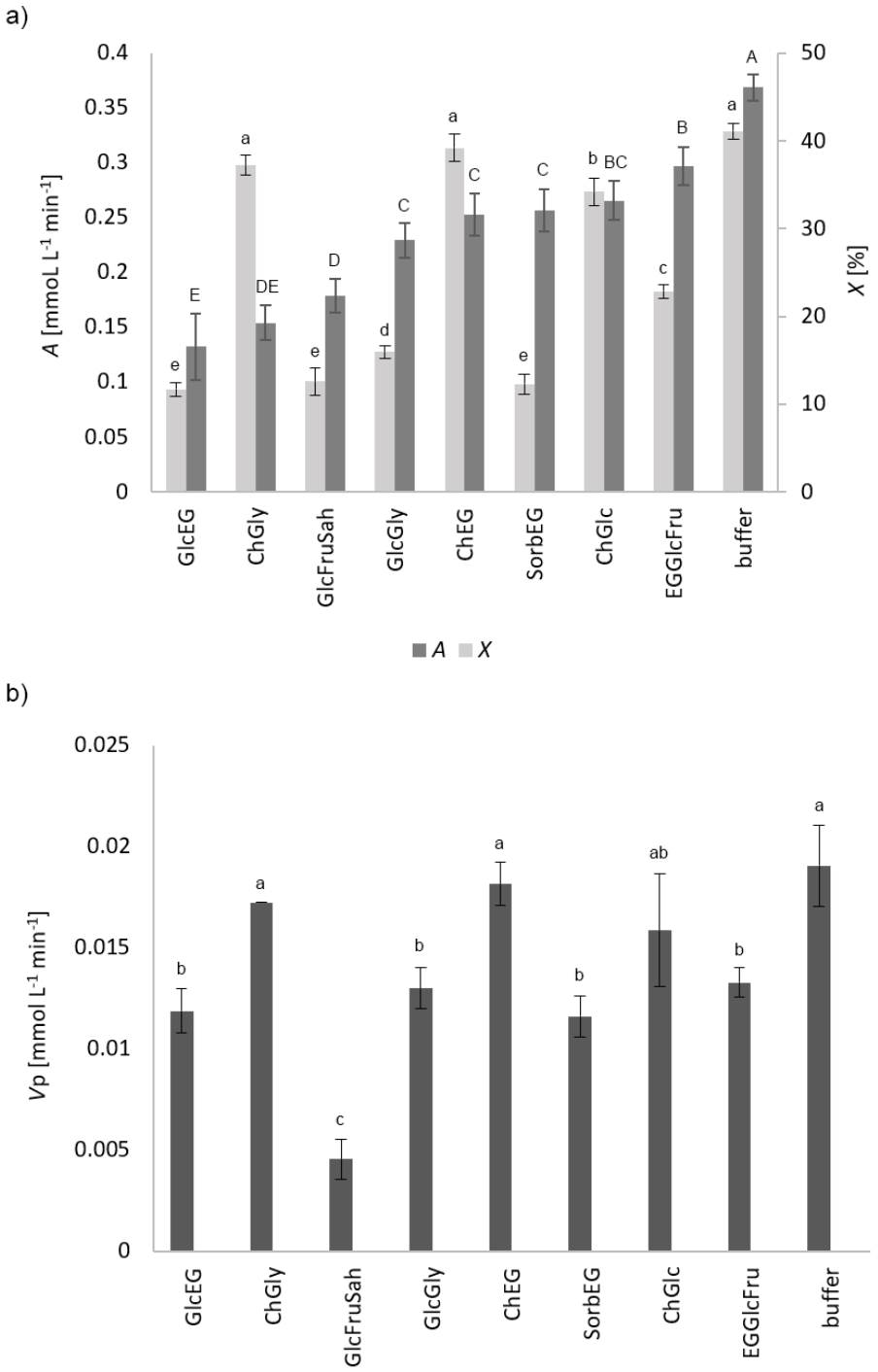


Figure 4.12. Initial reaction rates (*A*), conversion (*X*) (a) and productivity (*V*p) (b) of Novozym 435—mediated hydrolysis of (*R,S*)-1-phenylethyl acetate within different NADES (50 %, v/v) and buffer. Reaction conditions: 0.05 M (*R,S*)-1-phenylethyl acetate; 5 mg Novozym 435; 25°C; 1 mL. Each data point represents the mean of triplicate experiments; error bars represent the SD. Every group of results followed by nonidentical letters (a-e and A-E) indicate signifcant difference ($p < 0.05$) determined by Tukey's HSD test.

Results obtained indicated that lipase exhibit strong *R*-stereopreference regardless the NADES used (*ee*~99 %). On the other hand, measured initial reaction rates and productivity were proved to be dependent on NADES used. Initial reaction rate, conversion and productivity were in the range 0.1323- 0.369 mmoL L^{-1} min^{-1}, 11.69-41.14% and 0.00456-0.01905 mmoL L^{-1} min^{-1}.

The reaction productivity was not significantly different for ChEG, ChGly, ChGlc and buffer (p<0.005), the stability of enzyme in those solvents with different water content (10-50 %) at 25°C for 28 days was also considered prior to NADES selection (Figure 4.13.).

As shown on Figure 4.13., no differences in Novozyme 435 stability in the various solvents were observed at room temperature. The only exception was found for choline chloride:glycerol where immobilised *Candida antarctica* lipase B showed much higher stability (~80%, 28 days) compared to buffer (~40%, 28 days).

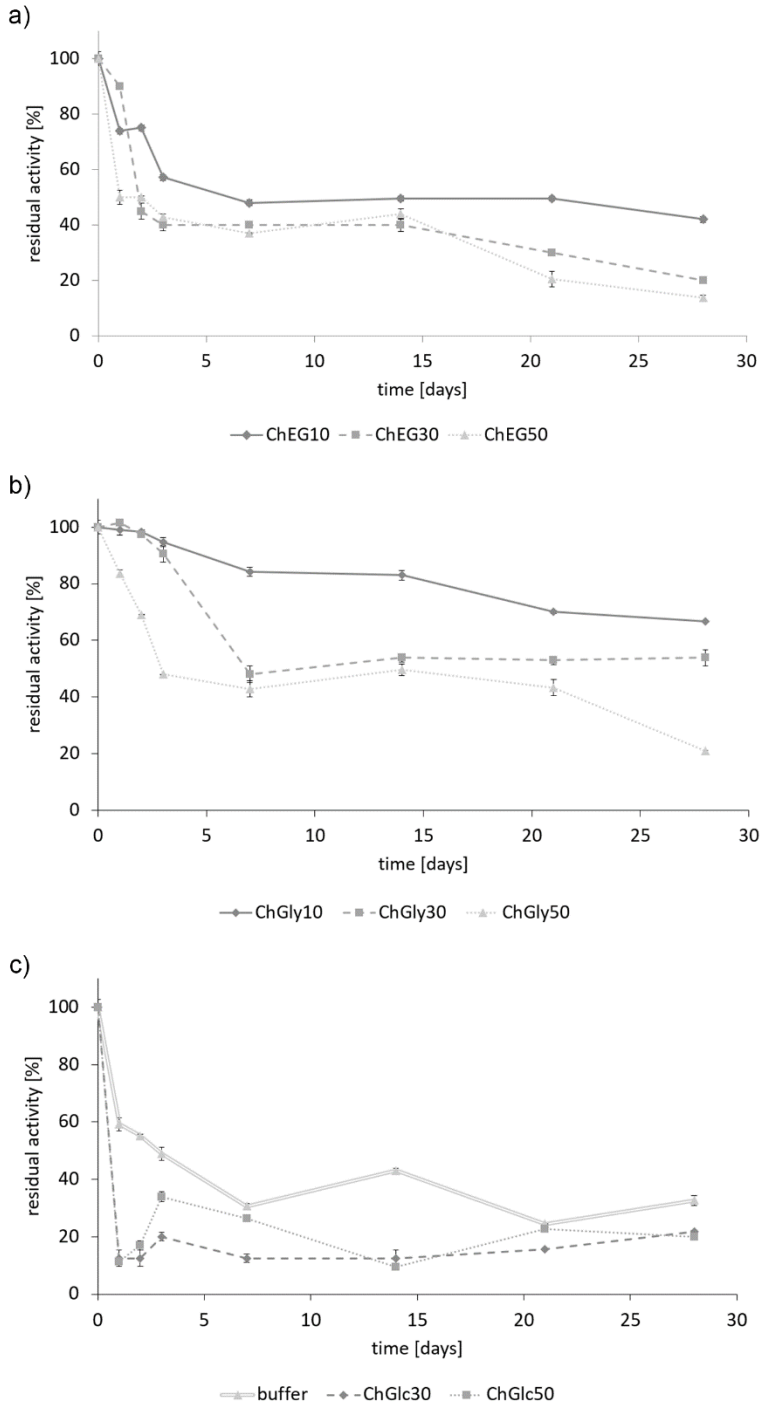


Figure 4.13. Relative activity of Novozym 435 after incubation in ChEG (a) ChGly (b), ChGlc, and buffer (c) at 25°C. Reaction conditions: 0.1 mol L^{-1} butyl acetate; 5 mg Novozym 435; 25°C; 1 mL of solvent. Each data point represents the mean of triplicate experiments; error bars represent the SD.

Further, deactivation constants were calculated (Table 4.15.).

Table 4.15. Deactivation constants (K_d) of Novozyme 435 under different inactivation solvents on 25°C during 30 days.

solvent	water content in solvent (%, v/v)	K_d/ day^{-1}
ChEG	10	0.0244 ± 0.0069
	30	0.0428 ± 0.0011
	50	0.0519 ± 0.0103
ChGly	10	0.0150 ± 0.0012
	30	0.0249 ± 0.0084
	50	0.0401 ± 0.0095
ChGlc	30	0.0215 ± 0.0303
	50	0.0388 ± 0.0422
buffer	-	0.0317 ± 0.0116

The rate of enzyme inactivation was the highest in ChEG50 and the lowest in ChGly10. Measured K_d values indicating decrease of enzyme-inactivation with higher content of NADES.

Based on experimental data on lipase behaviour in tested NADES and enzyme stability in NADES, ChGly was selected as the most promising solvent for the given reaction so it was used for further optimisation.

4.3.2. Laboratory process optimisation of the lipase-catalysed (*R*)-1-phenylethanol synthesis in ChGly

Based on preliminary data, the following independent variables for response surface methodology (RSM) in this study were selected: water content in NADES ChGly (10%-50%); reaction time (1-6 h); and temperature (20-60°C) for the highest impact on reaction conversion (Table 4.16.)

Table 4.16. Experimental matrix and values of observed response.

Run	t [h]	$w_{(H2O,NADES)}$ [%, w/w]	T [°C]	X [%]
1	3.5	10	20	20.02
2	3.5	50	60	50.52
3	3.5	50	20	24.90
4	6	50	40	42.03
5	3.5	30	40	35.99
6	1	30	60	36.65
7	3.5	10	60	41.06
8	6	30	20	27.11
9	3.5	30	40	35.71
10	1	10	40	21.44
11	1	50	40	24.20
12	3.5	30	40	35.75
13	6	10	40	31.58
14	6	30	60	55.28
15	1	30	20	19.74

* t -time, $w_{(H2O,NADES)}$- water content in NADES, T- temperature

The reaction conversion obtained varied from 19.09-55.28% (Table 4.16.).

Furthermore, the RSM model was evaluated and analysis of variance (ANOVA) was used to calculate the statistical significance of the model (Table 4.17.).

Table 4.17. Analysis of variance (ANOVA) of the modelled responses.

Source	*Conversion (Quadratic)*			
	Sum of squares	*Mean square*	*F-value*	*p-value*
Model	1640.42	182.27	906.56	< 0.0001
X_1	364.08	364.08	1810.83	< 0.0001
X_2	94.82	94.82	471.63	< 0.0001
X_3	1052.04	1052.04	5232.61	< 0.0001
$X_1 X_2$	14.79	14.79	73.57	0.0004
$X_1 X_3$	31.72	31.72	157.76	< 0.0001
$X_2 X_3$	5.25	5.25	26.13	0.0037
X_1^2	27.20	27.20	135.29	< 0.0001
X_2^2	39.90	39.90	198.47	< 0.0001
X_3^2	9.41	9.41	46.81	0.0010
Residual	1.01	0.20		
Lack of fit	0.96	0.32	13.81	0.0683
Pure error	0.046	0.02		
Total	1641.42			
R^2	0.9994			

$p < 0.01$ highly significant; $0.01 \leq p < 0.05$ significant; $p \geq 0.05$ not significan
X_1:time; X_2: water content in ChGly; X_3: temperature

Temperature, water content and reaction time had significant influence on reaction conversion (p<0.5). ANOVA showed that the model was significant, and lack of fot not significant, and can describe the influence of tested variables on converson (R^2=0.9994, p < 0.0001, F=906.56). The obtained results indicate the reliability of the developed model within the range of the studied variables.

The second-order polynomal equations for conversion are as follows:

$$Conversion\,(\%) == 35.81 + 6.75 * X_1 + 3.44 * X_2 + 11.47 * X_3 + 1.92 * X_1 * X_2 +$$

$$2.82 * X_1 * X_3 + 1.15 * X_2 * X_3 - 2.71 * {X_1}^2 - 3.29 * {X_2}^2 + 1.60 * {X_3}^2 \qquad [4.6.]$$

where X_1 is time, X_2 is water content in ChGly and X_3 is temperature.

The experimental data acquired were used for the creation of 3-D responce surface plots for each of the responses (Figure 4.14.)

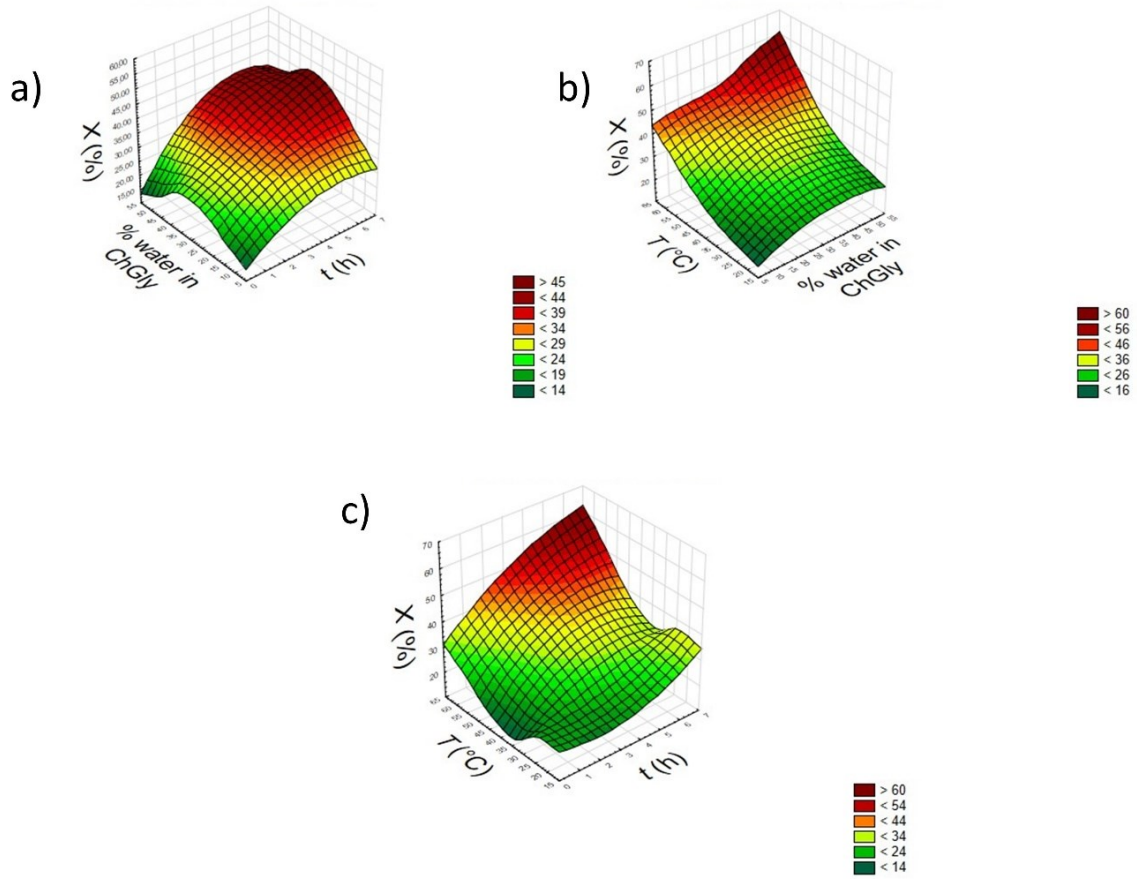


Figure 4.14. Response surface plots showing combined effect of water content in ChGly and reaction time (*t*) (a), temperature (*T*) and water content in ChGly (b) or temperature and reaction time (c) on reaction conversion (*X*).

The optimal values for the maximum response were obtained using the numerical tools od Design expert, and they were: *X* - 50.14%. The independent variables were: X_1=5 h 52 min, X_2= 47.75% of water in NADES ChGly and X_3=50.4°C. The verification and validity of the generated mathematical model were performed via the kinetic resolution of (*R,S*)-1-phenylethylacetate at these optimal conditions. After performing that experiment under the optimal conditions, *X* was 52.30%±1.11. It can be seen very close agreement between experimental and predicted value.

4.3.3. Product isolation and NADES recycling

Downstream separation of produced (*R*)-1-phenylethanol in ChGly were performed involved the removal of the biocatalyst from reaction media, NADES recycle and (*R*)-1-phenylethanol purification in 5 cycles (Figure 4.15.).

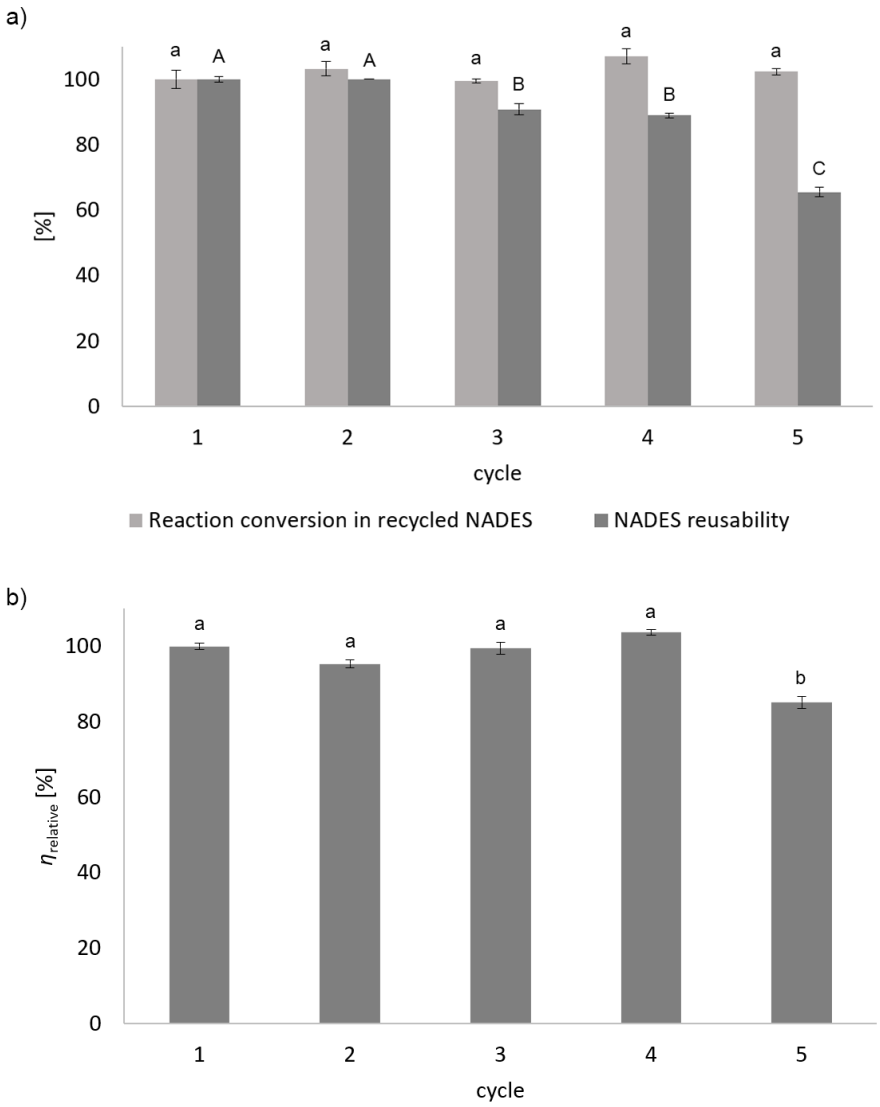


Figure 4.15. NADES reusability and reaction conversion in recycled NADES (a) and enzyme reusability (%) (b), of (*R*)-1-phenylethanol synthesis in ChGly with 47.75% of water. Reaction conditions: 0.05 M (*R,S*)-1-phenylethyl acetate; 5 mg Novozym 435; 40°C; 1 mL. Each data point represents the mean of triplicate experiments; error bars represent the SD. Every group of results followed by nonidentical letters (a-b and A-C) indicate signifcant difference ($P < 0.05$) determined by Tukey's HSD test.

At the end of the 5th cycle, relative yield of Novozyme 435 was 81.91%, while the *ee* (>99%) stayed on maximum in all of the 5 repeated batches. ChGly volume decreased 35% from the freshly synthesised solvent. Conversion and enantioselectivity of (*R,S*)-1-phenylethyl acetate hydrolysis performed in recycled ChGly was the same as in freshly synthesised NADES. Yield of product purification was 81.24 %.

4.3.4. Scale-up of the laboratory optimised biocatalysis

After laboratory scale optimisation, biocatalytic reaction was performed in half liter batch, under optimum condition from lab scale. A preparative-scale experiment proceeded as follows. Novozyme 435 was used for hydrolysis of 0.05 mol L^{-1} (*R,S*)-1-phenylethyl acetate in the overall reaction volume of 500 mL ChGly with 45% of water on 50.4°C for 5 h and 52 min. The conversion of produced (*R*)-1-phenylethyl acetate was 54.57 %. The enatioselectivity was the same as in laboratory scale (Table 4.18.).

Furthermore, downstream was also performed after preparative scale production of (*R*)-1-phenylethanol in ChGly, following the optimal conditions in laboratory scale. Yield of ChGly recyclation was 97%, while (*R*)-1-phenylethanol purification was 49.48%.

Table 4.18. Summary of up- and downstream results of biocatalytic process on laboratory and preparative scale.

	Upstream	Downstream	
	X [%]	η_{ChGly} [%]	$\eta_{(R)\text{-1-phenyethanol purification}}$ [%]
Laboratory scale	50.14	100-65.5	81.24
Preparative scale	54.57	97	49.48

5. DISCUSSION

Nowadays, the choice of a solvent in certain process not only depends on its chemical, and physical properties, but also on its environmental impact (e.g. ecotoxicity and biodegradability), sustainability (possibility of recycle and reuse) and process safety (e.g. flammability and volatility) (Chemat et al., 2012;). Therefore, interest for potential application of natural deep eutectic solvents (NADES) as a solvent and/or supporting medium, in a different process has increased significantly, due to their unique physical and chemical properties, and low environmental impact (Paiva et al., 2014; Paiva et al., 2018). One of the major attractions of NADES being an alternative to conventional organic solvents lies in the fact that the number of structural combinations encompassed by these solvents is tremendous, thus it is possible to design an optimal one for each specific application. NADES are very interesting for use in: (*i*) separation and isolation of various compounds and (*ii*) biocatalysis (Paiva et al., 2014; Paiva et al., 2018). In literature are a lot of succesfuly performed processes using NADES, but just a few of them are in pilot scale (Abbott et al., 2007). Commercialization of new technologies is always a high-risk. For this to be accomplished, it is essential to find some general rules for method development.

This thesis offers a steps towards the designing of extraction and biocatalysis processes with NADES. The aim was to prepare NADES and apply them in industrial interesting processes: (*i*) extraction of anthocyanins-reach extracts from wine industry waste and (*ii*) lipase-catalysed enantioselective preparation of industrial important chiral alcohol (*R*)-1-phenylethanol. Prepared polyphenolic extracts in NADES was also considered as ready-to-use in industry without downstream purification steps. According to economic demands, used solvent in industrial scale should be possible to recycle and since the NADES have very low vapour pressure this is a chalenge. Therefore, in both processes' possibilities of recyclation and reuse of NADES was tested.

5.1. Development of environmentally friendly method for extraction of anthocyanins from grape pomace with NADES and alternative energy sources

Recent literature has provided a number of examples of the NADES-mediated extraction of biologically active compounds, especially polyphenols (Bosiljkov et al., 2017; Cvjetko Bubalo et al., 2018; Jeong et al., 2015a; Zainal-Abidin et al., 2018). Polyphenols have successfully been extracted from industrial by-products, such as wine lees, red grape pomace, onions, olives, tomatoes, pear, and lemon-waste peels (Mouratoglou et al., 2016; Bosiljkov et al., 2017; Fernández et al., 2018). One of subgroup of polyphenols are anthocyanins.

Anthocyanins are important pigments responsible for flower, fruit, and vegetable colour, producing a complex variety of hues ranging from yellow to red through purple to blue. Recently, anthocyanins are becoming interesting in the scientific community also as health promoters (Giusti and Sigurdson, 2019). The rich biomass source of source of natural bioactive compounds (NBCs) and/or natural antioxidants due to high content of anthocyanins is grape pomace, wine production by-products. Food processing generates a substantial volume of solid organic by-products. Only small amounts of these by-products are up-graded or recycled and used usually for composting or even discarded in open areas potentially causing environmental problems. Therefore, the large quantity of agricultural and food production waste remains and represents a great challenge as well as opportunity for its commercial usage and valorization.

However, all published research data to date refer to the lab scale, and, to the best of our knowledge, there are no commercial NADES-based process in existence. NADES show great potential for industrial application thanks to their acceptable costs, the versatility of their physicochemical properties, simple and inexpensive preparation and low cytotoxicity (Panić et al., 2019).

In this part of research, the aim was to investigate the scale-up of NADES-assisted extraction and the efficient final separation of target compounds from the extraction media following the green extraction principles (Chemat et al., 2019). The experimental design entails the following steps: (1) NADES selection; (2) extraction method selection and parameter optimisation with scale-up; and (3) the recovery of the target compounds and NADES recycling. Several NADES have been prepared and fully characterised. The most promising NADES was selected for further optimisation according to physicochemical characteristics, anthocyanin extraction power and extract stability. The influence of MW and US, used alone or simultaneously, was also investigated. The parameters that influence the extraction process, such as time, water addition and power of alternative energy sources, MW and/or ultrasound, were also studied and optimised using the response surface methodology. Finally, the optimised method for the extraction of anthocyanins from grape pomace was performed on a larger scale (0.5 L), with NADES recycling.

5.1.1. NADES Selection

The first step in NADES selection is choosing the hydrogen bond acceptor and donor based on the properties of the target compounds, namely grape-pomace anthocyanins. Anthocyanins are highly polar compounds that are better soluble in water than in non-polar

solvents, whose chemical form and stability depend on pH value. Anthocyanins are prevalent in the flavylium cation form, which is stable at pH<2, while they change structure at increasing pH values, and are degraded at pH >7 (Cvjetko Bubalo et al., 2016b; Bosiljkov et al., 2017; Cheynier et al., 2012). The literature indicates that organic acid-based NADES can fully address the requirements of anthocyanin isolation and 8 different organic-acid based NADES were prepared and characterised (Table 4.1) (Bosiljkov et al., 2017; Cvjetko Bubalo et al., 2018, 2016; Jeong et al., 2015b; Radošević et al., 2016). All of the prepared solvents were polar, with polarity similar to water, and pH values were from 0.49-3.27. NADES were prepared with 25% (v/v) of water in order to decrease viscosity and improve mass transfer between the solid and liquid phases (Cvjetko Bubalo et al., 2018). Results were also compared to acidified aqueous ethanol as the most suitable and green conventional solvent (Makris, 2018).

The highest content of total anthocyanins (sum of identified anthocyanins by HPLC) was extracted with ChCit≈ChProMa≈EtOH, followed by ChMa≈BCit≈MaGlcGly≈ProMa≈BMa>MaGlc. Furthermore, values ranged from 0.28 to 0.92 mg g^{-1} dw, indicating great variation in NADES extraction efficiency (Figure 4.3.). Although polarity and pH values were previously highlighted as the most important solvent characteristics for anthocyanins extraction, no obvious correlation was observed (Radošević et al., 2016c). It seems that viscosity is the major property that influences extraction efficiency for organic acid based NADES since an obvious relationship was found; extraction efficiency increased as solvent viscosity decreased. Furthermore, the obtained anthocyanin profiles in extracts were in good agreement with literature; four anthocyanin-3-*O*-monoglucosides (delphinidin, petunidin, peonidin, malvidin), two acylated derivatives (malvidin- and peonidin-3-acetylmonoglucosides) and two coumaroyl derivatives (peonidin- and malvidin-3-(6-*O*-p-coumaroyl) monoglucosides) were observed, with malvidin-3-(6-*O*-p-coumaroyl) monoglucosides being most abundant (Kammerer et al., 2004; Teixiera et al., 2014). A comparison of conventional solvent and NADES highlighted that the latter showed higher extraction efficiency for anthocyanin-3-*O*-monoglucosides, while anthocyanin-3-(6-*O*-p-coumaroyl) monoglucosides were displayed improved extraction with acidified ethanol. The different selectivities of anthocyanin extraction with NADES and conventional solvents have already been published (Dai et al., 2016) (Table 4.2.). The stabilisation ability of NADES is correlated with strong hydrogen bonding between solutes and solvent molecules. This interaction decreases the movement of solute molecules, reducing their contact time with oxygen and the interface of NADESs and air, and consequently reducing oxidative degradation, which is the major degradation mechanism (Cvjetko Bubalo et al., 2018; Dai et al., 2016).

As the extraction efficiency was not significantly different ($p<0.005$) for ChCit, ChProMa and acidified aqueous ethanol, and the stability of anthocyanins in those solvents at -18°C, 4°C and 25°C for 60 days was also taken into account prior to NADES selection. This is especially interesting because extracts prepared with NADES can be considered safe for further application without expensive downstream purification (Radošević et al., 2016a) (Figure 4.4.). Generally, no differences in anthocyanin stability in the various solvents were observed at room temperature, while ChCit was found to possess the highest stabilising capacity at 4°C and -18°C. At 4°C, 70% of anthocyanins were degraded in ChProMa, 50% in acidified aqueous ethanol, while in ChCit it was only 14%. Similarly, it was noticed that only 10% of anthocyanins were degraded at -18°C in the ChCit-extract. Dai et al. (2016) have also noticed the higher stability of polyphenols in NADES than in conventional solvents.

Solvent cost is an important factor in scale-up development. The price of NADES is generally similar to that of organic solvents (~50 € kg^{-1}), but NADES structure can vary considerably. The cost of tested mixtures ranged from 15.02 € kg^{-1} (MaGlcGly) to 351.18 € kg^{-1} (ChProMa). Price of ChCit was 46.79 € kg^{-1}. An analysis of all relevant criteria (physicochemical characteristics, extraction efficiency, stability of anthocyanins and solvent cost) led to selection of ChCit, as the solvent for the isolation of grape-pomace anthocyanins, and it was thus used in further experiments.

5.1.2. Selection and optimization of the extraction method

After NADES selection, the eco-friendly extraction method was selected and optimised, according to 3rd principle of green extraction. Innovative technologies (US, MW, and UMAE) were considered as a means to reduce energy consumption and increase extraction efficiency. Non-conventional enabling technologies have found several potential applications in food industries thanks to their higher efficiency and energy savings (Barba et al., 2016). US can dramatically improve the extraction of a target component mainly through the phenomenon of cavitation. Meanwhile, MW heats the whole sample very quickly, inducing the migration of dissolved molecules. The simultaneous irradiation increases solvent penetration into the matrix, facilitates analyte solvation, and usually increases the solubility of target compounds (Leonelli and Mason, 2010).

Grape pomace-extracts in ChCit with 25% (v/v) of water were prepared using a range of techniques. Anthocyanin contents were extracted in the following order, highest to lowest, UMAE>MAE>UAE (Figure 4.5.). UMAE irradiation was therefore selected as the extraction method.

Process parameters were subsequently optimised using response-surface-methodology based experimental design. Software package experiments were designed using Design Expert 7.0.0., results were evaluated and the extraction method was optimised. Extraction time, power of MW irradiation and water content in the solvent were observed to have the highest impact on efficiency, and those parameters were optimised (MW power was tested from 100 – 300 W, water content in NADES from 30-50% v/v, and extraction duration from 10 – 15 min). MW power and extraction time were optimised in order to reduce energy consumption and prevent anthocyanin degradation. The water content in NADES was optimised in order to manage viscosity, but retain extraction efficiency.

Box-Behnken design was successfully used to evaluate the influence of UMAE process parameters; the response (content of total anthocyanins) and model design are shown in Table 4.3. The content of total anthocyanins ranged from 0.1 to 1.8 mg g^{-1} of dw. In order to obtain the significant effects of the independent variables on response, the designed experiments were performed with the central values for MW power, 200 W, an extraction time of 12.5 min and a water content in NADES of 30% (v/v) (Table 3.3.). Furthermore, the RSM model was evaluated and analysis of variance (ANOVA) was used to calculate the statistical significance of the quadratic model (Table 4.4.). The determination coefficient (R^2) of 0.9960 indicates that the quadratic regression model was a good fit for the extraction of grape-pomace anthocyanins with NADES in UMAE conditions. The F-value (135.46), with a very low *p*-value (<0.0001), of the model indicated that the quadratic model was statistically significant. According to the *p*-values, which were less than 5% for all variables, except X_2 X_3 and X_2^2, the variables had a statistically significant influence the content of grape-pomace anthocyanins (Table 4.4.).

Furthermore, 3D response-surface plots were created as function of two independent variables on fixed values (one independent and response) (Figure 4.6.). The significant influence that MW power had on total anthocyanin content is demonstrated in Figure. 4.6. (a, b). Total anthocyanin content increases with increasing MW power. Water content also had a significant effect on the extraction efficiency of total anthocyanins. The response-surface plots showed that total anthocyanin content increased with increasing water content, from 10 to 30%, while further increases led to decreases in total anthocyanin content, as has been previously noted (Bosiljkov et al., 2017). It seems that mass transfer between the solid and liquid phases is hampered by high solvent viscosity in NADES with 10% water, while anthocyanins are much less stable in NADES with higher water contents. Dai et al. (2014) have

also reported the similar relationship between water content in NADES and anthocyanin stability as was observed in this research, while Dai et al. (2013) and Nam et al. (2015) noticed that solvent viscosity is influenced by water content, and thus impacts upon mass transfer. MW and US irradiation also decrease the viscosity of NADES. Extraction time had a significant effect on total anthocyanin content. Increasing the extraction time led to decreased anthocyanin content, indicating that long extraction times and prolonged heating increase degradation (Cravotto et al., 2018).

The optimal values for the maximum response obtained using the Box-Behnken method was 1.8 mg g^{-1}_{dw}. The independent variables were: X_1= 300 W, X_2=600 s and X_3=30% of water in NADES (%). The verification and validity of the generated mathematical model were performed via the extraction of anthocyanins at these optimal conditions. After performing that experiment under the optimal conditions, extraction efficiency was 1.77 mg g^{-1}_{dw}.

5.1.3. From laboratory up to half-litre scale

According to results in laboratory scale, the aim of this part of dissertation was to scale-up the extraction method of high-value by-product from biomass, coupled with NADES and microwave ultrasound extraction. However, applicability of this technology in commercial processes is still in the initial phases of research and development. Only a few studies have reported scale-up of extraction processes involving NADES. When scale-up possibilities are planed, many factors need to be discussed and taken into consideration, including both the economic and environmental issues of NADES, as well as the technological aspect of exaction method. The costs of NADES are comparable with conventional solvents, and preparation is simple. Restrictive factor for using NADES as extraction solvents are some of the physicochemical properties as high viscosities of NADES and low vapour pressure.

UMAE is a cost-effective extraction technique for fast sample preparation and a new strategy for process intensifcation. UMAE reactors present several setup possibilities (Chemat and Cravotto, 2012; Leonelli and Mason, 2010). Although UMAE extraction can be achieved by inserting a non-metallic horn or US transducer into a MW oven, that only works on the lab scale (Cravotto and Cintas, 2007). A second possible instrument setup for UMAE on a larger scale is an US–MW flow reactor with sonication outside the MW oven. Cravotto et al. (2007) have successfully exploited flow reactors for the treatment of polluted water. However, it appears that pumping solid particles through the system for solid-liquid extraction is impossible. Sequential US and MW irradiation in batch reactors may therefore be the most

promising tool for industrial plant-extraction applications. Double irradiation can have synergistic effects on extraction processes and can double extraction efficiency; sonication can rupture cells, while MW promotes the release of the target compounds into the solvent (Chemat and Cravotto, 2012).

In this work, extraction was performed on a larger scale over 2 separate steps; US pretreatment, in the form of the sonication of grape pomace in ChCit (500 W, 5 min), before MW-assisted extraction (300 W, 10 min, 30% of water in NADES). Under such conditions, 1.58 ± 0.12 mg g_{dw}^{-1} of anthocyanins were extracted, which is equal to laboratory-scale results, indicating that the NADES process is promising for extraction on larger scales.

5.1.4. Recovery of anthocyanins and NADES recycling

Polyphenolic extracts that were prepared in NADES can be considered ready-to-use in the food and pharmaceutical industries, without the need for demanding and expensive downstream purification steps (Jeong et al., 2017; Radošević et al., 2016a). However, sometimes the final goal is to have pure target compounds for further industrial applications. Furthermore, solvents should be recycled after processing. Organic solvents that are used for extraction are usually recycled *via* distillation. Volatile organic solvents are a major source of industrial waste (Sheldon, 2016). On the other hand, NADES have very low vapour pressure (Cvjetko Bubalo et al., 2018, 2015a), making them very difficult to evaporate for the easy isolation of target compounds, which may be a problem for industrial applications. Among several possibilities for target-compound recovery and NADES recycling, herein adsorption chromatography using a macroporous resin were performed (Jeong, et al., 2015b; Ruesgas-Ramon et al., 2017). Adsorption chromatography was used in this work, with macroporous resin Sepabeads 825L as the stationary phase. From the grape pomace extract that was prepared with ChCit under optimum conditions on the lab scale, as mentioned above, anthocyanins were adsorbed onto a resin, NADES was eluted with deionised water, and anthocyanins were desorbed with acidified, aqueous ethanol (after 3 BV). The recovery yield of anthocyanins was 70.36% and the solvent recycling yield was 94.78%, but NADES still retained some colour. Since our final goal was the complete recovery of anthocyanins, as well as the recycling and reuse of the solvent for further extraction, a recovery yield of ~70% was not deemed to be satisfactory, and a number of ways to improve recovery were considered. Our hypothesis was that recovery yields would be higher if the bonds between NADES and target compounds were broken. As reported in Cvjetko Bubalo et al. (2015a), the bonding of molecules into the structure of NADES has been observed. Therefore, the grape-pomace extract

in ChCit was diluted to 80% of water prior to adsorption chromatography as the presence of > 50 % (v/v) of water ruptures the NADES structure (Yang, 2018). Under these conditions, anthocyanin recovery was highly efficient at ≈99.46 %, and was followed by highly efficient solvent recycling (yield 96.8 %). The cleanness of the NADES after recycling was checked using NMR spectrometry. The ^{1}H NMR spectra of freshly synthesised and recycled NADES were recorded for that purpose (Figure 4.7.) and no significant differences in ^{1}H NMR spectra were observed. Only the chemical shift characteristics of NADES were found on ^{1}H NMR spectra, indicating the cleanness of recycled NADES. This implies that NADES structures should be disturbed prior to recycling via dilution with > 50% of water (v/v) in order to release anthocyanins and make them available for better adsorption on macroporous resins.

Based on the results above, a method for anthocyanin recovery and NADES recycling was also performed on a pilot scale in an automatic double-column system that was presented in Cravotto et al., 2018. Anthocyanins from an extract that was prepared on a larger scale were recovered and the NADES was recycled. The recycling yield of ChCit in this system was 77.91 %, and anthocyanin recovery was ~ 90%. By comparison, Zhuang et al. (2017) and Wang et al. (2017) recovered 77.4-98.2% and 75.3-85.5% of polyphenols, respectively, on a macroporous resin from a plant extract that was prepared with NADES.

Furthermore, the regenerated solvent was then again used for the extraction of grape-pomace anthocyanins. Extraction efficiency with the recycled solvent was 1.42±0.11 mg g^{-1}, 11% less than that of freshly synthesised NADES. Similarly, an 8.1% lower amount of ginsenosides has been extracted using recycled NADES than with the original solvent system (Jeong et al., 2015b). However, our results indicate that the NADES extraction method is scalable as is anthocyanin recovery and solvent recycling. This study is a first step towards the implementation of this method on the industrial scale.

5.1.5. Evaluation of grape pomace NADES extract as ready-to-use extract for food and pharmaceutical application

Since NADES consist of simple, inexpensive and naturally occurring compounds with an implied high safety profile, it is assumed that extracts obtained by NADES may be directly used in products for human consumption without the need for expensive downstream purification steps (Choi et al., 2011; Dai et al., 2013b). Taking into account the potential applications of NADES and NADES-based extracts, it is necessary to further evaluate their biological activity (Panić et al., 2019b; Radošević et al., 2016c).

Human cell lines are already widely used in testing biological activity of compounds present in plants and plant's products. The application of *in vitro* cell cultures for this purpose is based on the fact that interaction of the test substance and biological systems occurs preferentially at the cellular level, and has an impact on the whole organism. With such approach a large number of samples, including crude extracts from screening phase, as well as pure fractions obtained during the isolation procedure, could be evaluated. Beyond that, the application of *in vitro* cell cultures is a good choice for preliminary screen of biological activities, which enables faster and more cost-effective studies compared to *in vivo* studies. The findings obtained by *in vitro* tests are surely valuable and can serve as guidelines for further *in vivo* and epidemiological studies of biological activity of plants, its compounds, and products (Radošević et al., 2016a).

Based on aforementioned, potential biological activity of polyphenolic extracts was evaluated by testing antiproliferative and antioxidant activity. *In vitro* cytotoxicity of prepared extracts was assessed by antiproliferation assay on two tumour cell lines, whereas for investigation of type of cell death and cell cycle arrest a flow cytometric analysis was applied. Antioxidant activity and possibility of extracts to protect cells from oxidative stress

5.1.5.1. Green polyphenolic extracts characterisation

Grape pomace extract is prepared according to conditions optimised in paragraph 5.1.2. Selected choline based NADES having organic acid as HBD is interesting due to biological activity of forming compounds itself (choline chloride and citric acid) which probably could enhance biological activity of plant extracts as well (Radošević et al., 2016c; Nam, et al., 2015). Choline is essential component of the human diet that is necessary for synthases of acetylcholine, membrane and signalling phospholipid, and functions as important methyl donor (Awwad et al., 2012). A few studies observed positive association between the dietary choline intake or plasma concentration and risk of some types of cancer (Xu et al., 2009; Zeisel, 2012).

The recommended daily requirement for choline has been set to 550 mg /day and 425 mg /day for non-pregnant women. The daily upper limit for adults is 3,500 mg per day which is the highest level of intake that is unlikely to cause harm (U.S. Food and Drug Administration, 2018). Similar, citric acid also possess various interesting biological activity including antioxidant, anti-inflammatory and antitumor effects (Chen et al., 2017). Citric acid is found in large quantity in many fruits and vegetables especially in citrus fruits and it is common food and drink additive widely used by food industry as chemical acidifier, flavouring agent and a preservative. It is general consider natural and healthy and for example, the Food and Drug Administration (FDA) does not pose limit for citric acid addition in food and drinks (Yoshikawa et al., 2011). However, to evaluate the NADES-based extract as possible ready to use in food and pharmaceutical industry without downstream purification steps, extracts from grape pomace were characterized and compared with those obtained with an aqueous solution of ethanol (70%, v/v) (Table 4.5.).

In general, considering solvent extractability, higher content of polyphenols from pomace was obtained with NADES ChCit. In grape pomaces extracts several polyphenolic compounds were identified and quantified including anthocyanins, gallic acid, (+)-catechin and quercetin-3-*O*-glucoside, with the highest content of anthocyanins. Obtained polyphenol profile in grape pomace extracts was in good agreement with previous studies (Teixiera et al., 2014). Anthocyanins profile is discussed in paragraph 5.1.1. Furthermore, in grape pomace-prepared extracts with NADES and aqueous ethanol, phenolic pattern was similar with some exception (Table 4.5.).

5.1.5.2. Biological evaluation of grape pomace extracts

Biological evaluation of grape pomace polyphenolic extracts, was conducted on two human tumour cell lines. Such *in vitro* preliminary screen of natural extracts is simple, inexpensive and rapid method for cytotoxicity assessment for a large number of samples in the range of different concentrations. Pure NADES and their forming compounds were also evaluated with regard to its *in vitro* cytotoxicity, having in mind that they also possess certain biological activities which could contribute the biological activities of plant extract. Ethanol extracts were also tested, as well as ethanol itself, since it is being used as referent solvent for polyphenolic extraction. Cytotoxicity of extracts (GPChCit, GPEtOH), extraction solvents (ChCit and EtOH) and NADES-forming compounds (ChCl and Cit) was evaluated by flow cytometry analyses on HeLa and MCF-7 cells. All samples were applied to cells in different volume ratio 1%, 5% and 10% (v/v) during 72 h (Figure 4.8.), where it was observed that

inhibitory effect of GPChCit, ChCit and Cit is almost the same no matter of applied volume ratio, while GPEtOH and Ch showed dose-dependent effect. EtOH as a solvent did not had impact on cell viability of HeLa and MCF-7 cells.

Extract obtained by ethanol (GPEtOH) have not had strong impact on cell viability of HeLa and MCF-7 cells, while rather low cell viability (from 12.19% to 37.61%) was observed in both cell lines treated with GPChCit, ChCit and Cit. Such grouping of results is probably related to low pH values of those samples (0.6 for ChCit, 0.91 for GPChCit, and 1.33 for Cit), for which it is all in common to contain citric acid. Citric acid as a HDB is surely responsible for these low pH values of NADES itself as well as extracts obtained by ChCit. The puffer system of used culture medium could not buffer it and, for example, the pH of the DMEM after addition of 5% of GPChCit was 2.83 what is still much lower than the optimal pH for cell culture cultivation. But it is known that *in vivo* many tumours have relatively acidic extracellular pH, while the intracellular pH of tumour cells remains normal due to the efficient maintenance of a large proton gradient across the membrane (Zanke et al., 1998). Considering that observed cytotoxicity of GPChCit in tumour HeLa and MCF-7 cells is probably mostly related to polyphenolic compounds in extracts, although synergistic effect of polyphenolics and low pH is possible, since there are data suggesting a link between induced cellular acidification and cell death (Harguindey et al., 2017). Furthermore, the comparison between extracts depending on type of extraction solvent used goes in support of the fact that polyphenols are most likely to be responsible for the notable antiproliferative effect of NADES-extracts. Grape pomace extract in ChCit had stronger cytotoxic effect on HeLa and MCF-7 cells than the one obtained by EtOH, what is in good correlation with HPLC analysis of individual polyphenolic compounds, measured TP and ORAC values (Table 4.5). TP and ORAC values were statistically higher ($p < 0.05$) for GPChCit than for GPEtOH, respectivly. Such result is in agreement with our previously published results on polyphenolic grape pomace extracts prepared by five choline chloride based NADES, where it was showed that the best performance concerning extraction efficiency and biological activities was obtained by ChMa i.e. NADES containing organic acid as HDB, which by itself possessed biological activity (Radošević et al., 2016c).

When it comes to observed significant antiproliferative effect of GPChCit, we wanted to elucidate whether inhibition of cell growth was related to cell cycle arrest or cell death and therefore we employed flow cytometry by Muse® Cell Analyzer and related assays to obtain quantitative data at the single cell level. Since there were no significant differences ($p<0.05$) in cytotoxicity results between two cell lines (Figure 4.9.) further experiments were done only on

HeLa cells treated with middle volume ratio (5%, v/v). According to the results presented on Figure 4.10. observed inhibition of cell growth by GPChCit could not be primary related to induction of apoptosis in HeLa cells, since there is no notable increase in number of apoptotic cells (early, late, total) when compared to control, untreated cells.

Apoptosis as a way of cell death has taken a central position in cell death investigations, especially those related with cancer research, although it becomes more and more evident that cells can also die by non-apoptotic mechanisms, such as autophagy, mitotic catastrophe and necrosis. Herein, significant difference between treated and control cells was observed only for those treated with GPChCit and GPEtOH, where percentage of dead cells is increased to 21.38% and 40.58%, respectively, meaning that those cells probably died by necrosis. Besides so-called accidental necrosis, newer literature also reports about necrosis-like programmed cell death which has been considered as a type of cell death, although its role in tumorigenesis and cancer treatment is still unclear (Park et al., 2013; Gali-Muhtasib et al., 2015).

The background of observed inhibitory effect in tumour cell lines could also be a delay in cell cycle, which is the most significant and fundamental processes in eukaryotic cells. As it could be seen at Figure 4.11. treatment of HeLa cells with extract prepared with NADES (GPChCit) significantly affected the distribution of cells in G0/G1 phase and reduced number of cells in S phase when compared to control cells. A slightly increase in G2/M population of cells, statistically significant when compared to control, was observed in the case of GPChCit and GPEtOH. These data suggest that inhibition of cell proliferation by NADES-extracts in HeLa cells is associated mainly with the induction of G0/G1 arrest, which allow cells to have time to trigger repair mechanisms or induce cell death pathways. Combined with obtained results on cell death this may indicate that observed G0/G1 arrest over time lead to necrosis. It is reported that the mechanisms of polyphenols activities are comprised of many different pathways from induction of apoptosis and cell cycle arrest, scavenging of free radicals, regulation of gene expression, and stimulation of the immune system (Dziedzic et al, 2017). Also, since it is known that different cell death mechanisms may lead one to another, it is rather difficult to determine the one dominantly responsible for certain observed outcome, like inhibition of cell growth, especially when dealing with natural plant extracts, which are mixtures of pro- and anti-apoptotic compounds, pro- and anti-oxidant compounds, etc.

As already mentioned, the most studied biological activity of polyphenolic compounds is their antioxidant activity, which surely contributes to the antioxidant capacity of related plant extracts (Xia et al., 2010). Since the major biological activity of polyphenolic compounds is related to its antioxidative activity, the antioxidative capacity of obtained plant extracts was

also measured by ORAC methods (Xia et al., 2010; Cioffi et al., 2010) and the obtained ORAC values of the extracts varied widely from 1229.65 to 2189.97 μmol TE g^{-1} dw, with statistically significant difference between those values. The highest value obtained for the GPChCit following by GPEtOH (Table 4.5.). Furthermore, we wanted to test the ability of grape pomace extracts to protect cells from induced oxidative stress. HeLa cells were pre-treated with extracts for 24 h and afterwards oxidative stress was induced by 100 μM H_2O_2 for further 4 h. Then the cell viability was determined and relative percentage of ROS positive cells was measured at the Muse® Cell Analyzer.

Results presented at Figure 4.11. showed statistically significant increase in HeLa cell viability when pre-treatment was done by GPChCit (63.88%) compared with H_2O_2 alone (44.87%). Increase in cell viability is in good correlation with significant decrease in the percentage of ROS(+) cells obtained in GPChCit pre-treated cells. Reduction of ROS(+) cells from 11.77% in H_2O_2 only sample to 1.63% in sample pre-treated with GPChCit confirms its antioxidant potential measured by ORAC method. Ethanol extract have not shown ability to protect cells from oxidative stress. Several studies tried to answer which polyphenolic compound is mainly responsible for antioxidant activities of specific plant, but consistent relationship between polyphenolic compounds and antioxidant capacity was not found. Besides, many studies indicate synergistic effects between individual classes of polyphenolics which could be related to their antioxidant capacity, rather than individual polyphenolic compound by itself (Teixiera et al., 2014; Shahidi and Ambigaipalan, 2015; Mao et al., 2017; Liu et al., 2018) so considering that plant extracts are probably better choice than pure polyphenolic compounds.

Considering all data presented, antioxidative activity of GPChCit extract is advantageous for many possible applications in food and probably pharmaceutical industry. Considering food industry, Durand et al. (2017) showed that formulation of antioxidants in NADES could greatly improve their activity in ROS inhibition, so it could be anticipated that such natural extract can serve as food preservative even better than currently known antioxidants. Application of extracts obtained by NADES in pharmaceutical industry is based on the fact that NADES improve absorption and bioavailability of active compounds (Faggian et al., 2016) and therefore could serve as a delivery agent or administration vehicle. Furthermore, it is already well known that grapes and its products have anticancer activity (Xia et al., 2010) so results obtained herein on human tumour cell lines confirms antitumor potential of polyphenolic extracts, but since there is still a debate should cancer treatments focus on lowering ROS levels to prevent

signalling or increasing ROS to selectively kill cancer cells (Schieber and Chandel, 2014), further studies in that direction are needed.

Taken all together, presented results indicate that selected environmentally friendly extraction method extract higher content of polyphenolics from food by-products than classical extraction with EtOH as a solvent. Further, studying biological activity of prepared extract, stronger cytotoxic effect on tumour cell lines of grape pomace extracts prepared with ChCit than extract prepared with EtOH was observed. Those results indicating potential antitumor activity of prepared NADES extract. Flow cytometry analysis revealed that necrosis is a possible mechanism behind the antiproliferative effect of prepared extract. Antioxidative activity and possibility to protect the cells from oxidative stress was also observed. Therefore, prepared NADES extract could be considered as ready-to-use in food and pharmaceutical industry without demanding and expensive downstream purification steps.

5.2. Development of environmentally friendly process for the synthesis of (*R*)-1-phenyl ethanol using lipase in NADES

Nowadays, single-enantiomer drugs have become the standard in pharmaceutical companies when working with compounds featuring stereogenic centers, mobilizing chemical and biotechnological industry to develop versatile methods for production of biologically active chiral compounds of high enantiomeric purity (Nguyen et al., 2006). Namely, enantiomers of a single chiral drug may differ significantly in their biological effect (sometimes even opposite), as well as in their bioavailability, rate of metabolism, metabolites, excretion, potency and selectivity for receptors, transporters and/or enzymes, and toxicity. These enantiomerically pure compounds could be synthesized by traditional synthesis methods using organometallic compounds as catalysts, however, poor enantioselectivity and residues of metal catalysts in final products makes this approach rather undesirable. Overview of current literature on chiral drug production and formulation reveals exciting, new, efficient and green approach: the use of NADES as medium for (bio)chemical preparation of chiral biologically active compounds and as auxiliaries in development of chiral drug delivery system (Paiva et al., 2018; Vanda et al., 2018).

In this part of the research, the aim was development of NADES-assisted protocol for synthesis of (*R*)-1-phenylethanol that would be interesting both from economical and ecological point of view. For that purpose, lipase catalyzed the kinetic resolution of (*R,S*)-1-phenylethyl acetate within NADES was performed. When scheming this research, major points were governed in designing a biocatalytic involving NADES: (*i*) screening of NADES for optimal

enzyme performance; (*ii*) optimization of the biocatalytic protocol; (*iii*) preparative scale – up- and downstream of (*R*)-1-phenylethanol synthesis.

5.2.1. NADES selection for optimal lipase-catalysed performance for (*R*)-1-phenylethanol synthesis

In this work enantioselective hydrolysis of (*R, S*)-1-phenylethyl acetate to (*R*)-1-phenylethanol by immobilized *Candida antarctica* lipase B (Novozym 435) within 8 different cholinium-based or cholinium-free NADES was conducted (Table 4.6.). Enantioselective hydrolysis was also performed in 0.025 M potassium phosphate buffer as a reference solvent. Blank experiments without the enzyme were performed in all tested NADES as well, and no conversion was observed within reaction times used in this study (data not shown).

Furthermore, NADES selected were weakly acidic to neutral (Table 4.6.) since it is well known that lipase is able to perform reactions under mild conditions (pH and temperature), with remarkable chemo-, regio-, and stereoselectivity. As for price of NADES, estimated price based on cost of raw materials varies from approx. 5.57 € kg^{-1} for GlcGly up to 656.89 € kg^{-1} for SorEG, simmilar as conventional solvents (Table 4.6.).

Results obtained indicated that lipase exhibit strong *R*-stereopreference regardless the solvents used (ee~99 %). This stereopreference is advantageous as it allows the direct production of the desired *R*-alcohol without further chemical manipulation. Rios et al. (2016) also noted enantioselectivity of the lipase to hydrolyze only (*R*)-phenylethyl acetate in a mixture of this racemic ester. On the other hand, measured initial reaction rates (k_o), conversion (X) and productivity (V_p) were proved to be dependent on NADES used. Values for k_o, X and V_p were in the range 0.1323- 0.369 mmoL L^{-1} min^{-1}, 11.69-41.14% and 0.00456-0.01905 mmoL L^{-1} min^{-1}, respectively (Figure 4.12.). The highest k_o was observed in conventional solvent, potassium-phosphate buffer. It is not surprising that k_o rate in buffer is higher than in NADES, since Durand et al. (2012) concluded that lipase in NADES needs time to adopt its final structure for what decreasing of activity in first hours of incubation is present.

Improvement in X and V_p values in the optimal NADES (ChGly, ChEG, ChGlc) in regars to reference solvent (buffer) was not observed, however stability of the enzyme in NADES was significantly enhanced in certain NADES (Figure 4.13 & 4.14.). The storage stability of Novozyme 435 was monitored at 25°C for 28 days in ChGly, ChEG, ChGlc and buffer (Figure 4.14.). ChGlc with 10% of water was to viscous for manipulation, and experiments in that solvent was not performed. In summary, no differences in Novozyme 435 stability in the

various solvents were observed at room temperature. The only exception was found for ChGly where immobilised *Candida antarctica* lipase B showed much higher stability (~80% of initial activity, 28 days) compared to buffer (~40% of initial activity, 28 days) (Figure 4.13.), implying that NADES could serve as stabilizing medium for lipase. Simmilar, when monitoring the stability of *Candida antarctica* lipase B immobilization with cross-linking aggregates, Guajardo et al. (2019) have also noticed the higher stability of enzyme in ChGly than in other NADES (ChEG and ChU). The results regarding storage stability of Novozyme 435 in ChEG are in agreement with the paper from Cvjetko Bubalo et al. (2015a), where about 50% decrease of initial lipase activity was measured after 3 days incubation of the enzyme in ChEG, nevertheless extending the incubation time up to 15 days did not result in any significant enzyme activity loss.

Further, inactivation rate constants (K_d) were calculated (Table 4.15.). The rate of enzyme inactivation was the highest in ChEG with 50% of water (0.0519 day^{-1}) and the lowest in ChGly with 10% of water (0.0150 day^{-1}). Measured K_d values indicating decrease of enzyme-inactivation with higher content of NADES. Dependence of water content in NADES on Novozyme 435 deactivation was also reported by (Cvjetko Bubalo et al., 2015a) when studying the influence of water content in ChGly for *Candida antarctica* lipase B-catalyzed reaction of acetic anhydride with 1-butanol to give ester butyl acetate.

Based on experimental data on lipase behaviour in tested NADES, and enzyme stability in NADES, ChGly was selected as the most promising solvent for the given reaction so it was used for further optimisation.

5.2.2. Laboratory scale process optimisation

Biocatalytic process has two parts which should be optimised: upstream (product development) and downstream (product purification). After choosing the optimal NADES, within pH, polarity and viscosity of a medium were optimised, next step is optimization of the biocatalytic protocol. Searching the literature, it is evident that amount of water in NADES, temperature, enzyme to substrate ration, pH value, and reaction time have influence on the enzyme activity. Water in NADES (>60%, w/w) may damage strong hydrogen-bonding network of NADES (Cheng and Zhang, 2017; Khandelwal et al., 2016), but on the other hand, lower amounts of water (approx. 30%) could negatively influence hydrolysis. Beside water, well known process parameter that need to be optimized are reaction temperature and substrate(s) concentration (Pätzold et al., 2019). Thus, based on preliminary data and the current

literature, the following independent variables for RSM analysis in this study were selected: reaction time (1-6 h); water content in ChGly (10-50%); and temperature (20-60°C). Enzyme to substrate ratio was fixed to 1.3 according to preliminary results (data not shown).

Above mentioned process parameters were subsequently optimised using RSM based experimental design (Box-Behnken design). Software package experiments were designed using Design Expert 7.0.0. and results were evaluated and the conversion of kinetic resolution of (*R,S*)-1-pnenylethylacetate to (*R*)-1-phenylethanol was optimised. The reaction conversion obtained varied from 19.09-55.28% (Table 4.16.). The lowest conversion for response investigated was obtained using the ChGly with 10% water, with a time of 3 h and 30 min, and temperature of 20°C.

Furthermore, the RSM model was evaluated and analysis of variance (ANOVA) was used to calculate the statistical significance of the model. The model for independent variable was selected according to determination and adjusted coefficiet (R^2) and the model *p* value (Table 4.17.). The results show that the independent variables had a significant effect ($p<0.001$). Also, all four variables had a positive coefficient, according to with an increase in specific variable led to an increased conversion. It is important to mention that ANOVA also showed the significance of both quadratic and interaction factors.

ANOVA showed good agreement between model predicted data and experimental data with determination coefficient of R^2=0.9994, $R^2_{adjusted}$=0.9983 and with no significant lack of fit at $p > 0.05$. The determination coefficient indicated that proposed model was able to explain 99.94% of results. Results also showed that the model used to fit response variable was significant (< 0.0001) and suitable for description of the relationship between independent variables and response. F-test suggested that model had a very high model *F*-value ($F = 906.56$), indicating this model was highly significant.

The experimental data acquired were used for the creation of three-dimensional response–surface plots for each of the responses (Figure 4.14.). These response–surface plots show that the conversion of hydrolysis of (*R, S*)-1-phenylethyl acetate to (*R*)-1-phenylethanol increased with reaction temperature and reaction time. On the other hand, the water content in the ChGly had a double effect on each response. The increase in water content from 10% to 45% in the ChGly showed increased conversion, while a further increase led to decreased reaction conversion. This once again indicates the importance of water content optimisation when applying NADES as reaction solvent. According to developed RSM model the optimum conditions for conversion were time of 5 h and 52 min, water content of 47.74% and

temperature of 50.4 °C. Model predicted the conversion of 50.14 % as optimum. After performing that experiment under the optimal conditions, *X* was 52.30 ± 1.11%. It can be seen very close agreement between experimental and predicted value.

Since the major cost in biocatalytic process in industry lies in downstream purification (Asenjo and Andrews, 2008), the last step in designing efficient biocatalytic process involving NADES is product isolation and purification, enzyme and NADES recycling and reuse. Therefore, downstream separation of (*R*)-1-phenylethanol from ChGly were performed. When performing biocatalysis in organic solvent, solvent is usually evaporated and final product is further purified. On the other hand, low vapour pressure of NADES makes these solvents very difficult to evaporate, which may be a problem for industrial applications (Cvjetko Bubalo et al., 2015b). Only a few research groups have reported a downstream separation of product after biocatalytic process in NADES (Jiang et al., 2019; Maugeri et al., 2012; Panić et al., 2018). Within this research, obtained *R*-alcohol, and unreacted (*S*)-1- phenylethyl acetate were isolated with extraction using ethyl acetate as the renewable solvent. Prior, Novozyme 435 was removed with filtration. (*R*)-1-phenylethanol and unreacted (*S*)-1-phenylethyl acetate from organic fraction were separated on silica gel column using *n*-heptan:ethyl acetate (4:1) as mobile phase (Figure 4.15a). Yield of product separation was 81.24%.

After removing the product and unreacted substrate, NADES was recycled. Further, recycled ChGly with 47.74% of water was reused for another round of (*R*)-1-phenylethanol production (Figure 4.16.a). The yield of the NADES ChGly recycled 5 times decreased 35% from the freshly synthesised solvent, caused by downstream procedure. Hydrolysis was also performed in recycled NADES, and the conversion and enantioselectivity in the recycled ChGly with 47.75% (v/v) of water was simmilar as in freshly synthesised NADES.

After the isolation and purification of (*R*)-1-phenylethanol at RSM-optimised conditions (at millilitre scale), Novozyme 435 was also recycled and reused. Novozyme 435 was washed with ethyl acetate and water, dried, and used again for the kinetic resolution of (*R,S*)-1-phenylethyl acetate in (*R*)-1-phenylethanol in fresh ChGly. The procedure was repeated 4 times (Figure 4.15b.). At the end of the 5th cycle, relative yield of Novozyme 435 was 81.91%, while the *ee* (>99%) did not decrease in all of the 5 repeated batches. Liang et al. (2012) also noticed similar effect studying recyclation of Novozym 435 in buffer-based media, where *ee* value did not change through 11 cycles, and relative activity was 70% after 11th cycle. Opposite, a slightly larger decline in Novozyme 435-activity reported Guajardo et al. (2017) where enzyme activity was 47 % after just 5th cycle of recyclation in 92% (v/v) ChGly.

5.2.3. Preparative scale (up and down)

After laboratory scale optimisation, biocatalytic reaction was performed in half liter batch reactor, under optimum condition from lab scale, afterwards, downstream process was also performed following the conditions in laboratory scale.

A preparative-scale up-stream experiment proceeded as follows. Novozyme 435 was used for hydrolysis of 0.05 mol L^{-1} (*R,S*)-1-phenylethyl acetate in the overall reaction volume of 300 mL ChGly with 45% of water on 50.4°C for 5 h and 52 min. The reaction conversion was 54.57%, similar as in laboratory scale. The enantioselectivity was the same as in laboratory scale (Table 4.18.). From the literature it seems that scalability of NADES-assisted biocatalysis have been performed only on preparative scale (up to 500 mL). Xu et al. (2015) carried out the reduction of 3-chloropropiophenone catalyzed by immobilized *Acetobacter* sp. CCTCC M209061 cells in ChU- containing system on a 500 mL scale under the optimal reaction conditions from laboratory scale. Yield (82.3%) was lower than that obtained on the 5.0 mL scale (93.3%) and the product *ee* was still high as in laboratory scale. Panić et al. (2018) scaled-up reduction of 1-(3,4-dimethylphenyl)ethanone (DMPA) by S. *cerevisiae* in ChGly to 300 mL from laboratory scale. Yield of reduction in bigger scale was 89.91%, the similar as in millilitre scale.

Furthermore, yield of purified (*R*)-1-phenylethanol was 49.48% ~ 30% lower than lab scale. NADES recyclation was 97%. Panić et al. (2018) also performed downstream process after preparative scale up and the reaction yield was quite lower or similar to millilitre scale, as in our case.

To conclude, highly-efficient and eco-friendly method for preparation of (*R*)-1-phenylethanol with lipase in NADES was developed. After laboratory scale optimisation, production of (*R*)-1-phenylethanol was also succesfuly performed with the simmilar efficiency on preparative scale what makes this process very interesting for future industrial application. ChGly was succesfully recycled and reused after biocatalysis. Special emphasis herein should be given to the observed enhanced stability of Novozyme 435 in ChGly. This contribution can be valuble from economic point of view for recycle and reuse of enzyme as well as performing of integrated processes.

6. CONCLUSIONS

In this work, prepared and characterised were natural deep eutectic solvents (NADES) and were applied in the extraction of anthocyanins from grape pomace and (*R*)-1-phenylethanol synthesis with *Candida antarctica* lipase B. According to results, following conclusion can be drawn:

1. 15 NADES were prepared and characterized were. The structure of NADES had influence on physicochemacal properties.
2. Eight prepared NADES were screened for the highest extraction efficiency of anthocyanins from grape pomace. Based on an analysis of all observed criteria (physicochemical characteristics, extraction efficiency, stability of anthocyanins and solvent cost) choline chloride:citric acid (ChCit) was selected as the solvent for the extraction of grape-pomace anthocyanins.
3. The effects of different extraction methods have been investigated in order to maximise anthocyanin extraction yield. The best conditions were found to be: simultaneous ultrasound/microwave-assisted extraction (UMAE). Process parameters of extraction were optimised using response surface methodology and the optimal conditions were: microwave (MW) power - 300 W, ultrasound (US) power - 50 W, time - 10 min and water content in ChCit - 30% (v/v) of water. Therefore 1.8 mg g_{dw}^{-1} of anthocyanins were extracted.
4. Based on laboratory scale extraction, extraction was performed on a larger scale over 2 separate steps: US pretreatment, in the form of the sonication of grape pomace in ChCit (500 W, 5 min), before MW-assisted extraction (300 W, 10 min). Therefore 1.58 mg g_{dw}^{-1} of anthocyanins were extracted, similar to lab-scale.
5. Anthocyanins were efficiently recovered from ChCit-based extract using adsorption chromatography with Sepabeads825L as stationary phase, while NADES was recycled and reused.
6. Evaluating biological activity of prepared extract, stronger cytotoxic effect on HeLa and MCF-7 tumour cell lines were observed with grape pomace extracts prepared with ChCit than extract prepared with EtOH. With flow cytometry analysis, delay in cell cycle as possible mechanism of antiproliferative effect of prepared extracts was determined. Antioxidative activity and possibility to protect the cells from oxidative stress was also observed with prepared ChCit-based grape pomace extract.

7. Seven prepared NADES were screened for the for lipase catalysed synthesis of (*R*)-1-phenylethanol. Based on experimental data on lipase behaviour in tested NADES. NADES characteristics and enzyme stability in NADES, choline chloride:glycerol (ChGly) was selected as the most promising solvent for the given reaction.
8. Response surface methodology was used for biocatalytic process parameters optimisation, resulting the optimum conditions for the highest conversion of (*R*)-1-phenyethyl acetate in (*R*)-1-phenylethanol: time of 5 h and 52 min, water content of 47.74% and temperature of 50.4°C.
9. Based on laboratory scale biocatalytic process, (*R*)-1-phenylethanol synthesis was performed in half liter batch reactor, under optimum condition from lab scale, where reaction conversion was similar as in mililiter scale- biocatalytic reaction.
10. Produced (*R*)-1-phenylethanol was successfully isolated and purified from reaction mixture using liquid-liquid extraction with ethyl acetate, and ChGly was recycled and reused. Further, (*R*)-1-phenylethanol and unreacted (*S*)-1-phenylethyl acetate were purified on silica gel column. Biocatalyst was also successfully recycled and reused in 4 cycles.
11. This dissertation formulates the postulates for the development of eco-friendly technology with natural deep eutectic solvents and their scale-up. The knowledge gained from this research contributes to the development of new, competitive and environmentally friendly industrial processes.

7. LITERATURE

Abbott, A.P., Boothby, D., Capper, G., Davies, D.L., Rasheed, R.K. (2004) Deep Eutectic Solvents formed between choline chloride and carboxylic acids: Versatile alternatives to ionic liquids. J. Am. Chem. Soc. **126**, 9142–9147.

Abbott, A.P., Capper, G., Davies, D.L., Rasheed, R.K., Tambyrajah, V. (2003) Novel solvent properties of choline chloride/urea mixtures. *Chem. Commun.* **1**, 70-71.

Abbott, A.P., Cullis, P.M., Gibson, M.J., Harris, R.C., Raven, E. (2007) Extraction of glycerol from biodiesel into a eutectic based ionic liquid. *Green Chem.* **9**, 868.

Abranches, D.O., Larriba, M., Silva, L.P., Melle-Franco, M., Palomar, J.F., Pinho, S.P., Coutinho, J.A.P. (2019) Using COSMO-RS to design choline chloride pharmaceutical eutectic solvents. *Fluid Phase Equilib.* **497**, 71–78.

Anastas P., Eghbali N. (2010) Green chemistry: principles and practice. *Chem. Soc. Rev.* **39**, 301–312. Andler, S.M., Wang, L.S., Rotello, V.M., Goddard, J.M. (2017) Influence of Hierarchical Interfacial Assembly on Lipase Stability and Performance in Deep Eutectic Solvent. *J. Agric. Food Chem.* **65**, 1907–1914.

Asenjo, J.A., Andrews, B.A. (2008) Mini-review Challenges and trends in bioseparations. *Chem. Eng.* **120**, 117–120.

Awwad, H. M., Geisel, J., & Obeid, R. (2012) The role of choline in prostate cancer. *Clin. Biochem.* **45**, 1548–1553.

Bakirtzi, C., Triantafyllidou, K., Makris, D.P. (2016) Novel lactic acid-based natural deep eutectic solvents: Efficiency in the ultrasound-assisted extraction of antioxidant polyphenols from common native Greek medicinal plants. *J. Appl. Res. Med. Aromat. Plants* **3**, 120–127.

Barba, F.J., Zhu, Z., Koubaa, M., Souza, A. De, Ana, S., Orlien, V. (2016) Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: A review. *Trends Food Sci. Technol.* **49**, 96-109.

Bogaars, R.A. (2015) Exploring Commercial Applications of Natural Deep Eutectic Solvents (Master thesis). Bosiljkov, T., Dujmić, F., Cvjetko Bubalo, M., Hribar, J., Vidrih, R., Brnčić, M., Zlatic, E., Radojčić Redovniković, I., Jokić, S. (2017) Natural deep eutectic solvents and ultrasound-assisted extraction: Green approaches for extraction of wine lees anthocyanins. *Food Bioprod. Process.* **102**, 195–203.

Chanioti, S., Tzia, C. (2018) Extraction of phenolic compounds from olive pomace by using natural deep eutectic solvents and innovative extraction techniques. *Innov. Food Sci. Emerg. Technol.* **48**, 228–239.

Chemat, F., & Cravotto, G. (2012) Combined Extraction Techniques, In N. Lebovka, E. Vorobiev, & F. Chemat (Eds.), Enhancing Extraction Processes in the Food Industry (pp. 173-195). CRC Press Taylor & Francis Group: New York.

Chemat, F., Abert-vian, M., Fabiano-tixier, A.S., Strube, J., Uhlenbrock, L., Gunjevic, V., Cravotto, G. (2019) Green extraction of natural products . Origins , current status , and future challenges. *Trends Anal. Chem*. **118**, 248–263.

Chemat, F., Rombaut, N., Sicaire, A.G., Meullemiestre, A., Fabiano-Tixier, A.S., Abert-Vian, M. (2017) Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrason. Sonochem.* **34**, 540–560.

Chemat, F., Vian, M.A., Cravotto, G. (2012) Green extraction of natural products: Concept and principles. *Int. J. Mol. Sci.* **13**, 8615–8627.

Chen, X., Lv, Q., Liu, Y., & Deng, W. (2017) Effect of Food Additive Citric Acid on The Growth of Human Esophageal Carcinoma Cell Line EC109. *Cell J.* **18**, 493–502.

Chen, Y., Zhang, W., Zhao, T., Li, F., Zhang, M., Li, J., Zou, Y., Wang, W., Cobbina, S.J., Wu, X., Yang, L. (2015) Adsorption properties of macroporous adsorbent resins for separation of anthocyanins from mulberry. *Food Chem.* **194**, 712–722.

Cheng, Q.-B., Zhang, L.-W. (2017) Highly Efficient Enzymatic Preparation of Daidzein in Deep Eutectic Solvents. *Molecules* **22**, 186.

Choi, Y.H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I.W.C.E., Witkamp, G.-J., Verpoorte, R. (2011) Are Natural Deep Eutectic Solvents the Missing Link in Understanding Cellular Metabolism and Physiology? *Plant Physiol.* **156**, 1701–1705.

Clark, J., Macquarrie, D. (2002) Handbook of Green chemistry and technology, Blackwell Science, Cornwall.

Clarke, C.J., Tu, W., Levers, O., Bro, A., Hallett, J.P. (2018) Green and Sustainable Solvents in Chemical Processes.

Cravotto, G., & Cintas, P. (2007) The Combined Use of Microwaves and Ultrasound: New Tools in Process Chemistry and Organic Synthesis. *Chem. Eur. J.* **13**, 1902-1909.

Cravotto, G., Mariatti, F., Gunjevic, V., Secondo, M., Villa, M., Parolin, J., Cavaglià, G. (2018) Pilot Scale Cavitational Reactors and Other Enabling Technologies to Design the Industrial Recovery of Polyphenols from Agro-Food By-Products, a Technical and Economical Overview. *Foods* **7**, 130.

Cravotto, Giancarlo, Stefano Di Carlo, Massimo Curini, V.T. and C.R. (2007) A new flow reactor for the treatment of polluted water with microwave and ultrasound. *J. Chem. Technol. Biotechnol.* **82**, 1115–1121.

Cvjetko Bubalo, M., Ćurko, N., Tomašević, M., Kovačević Ganić, K., Radojčić Redovniković, I. (2016b) Green extraction of grape skin phenolics by using deep eutectic solvents. *Food Chem.* **200**, 159–166.

Cvjetko Bubalo, M., Jurinjak Tušek, A., Vinković, M., Radošević, K., Gaurina Srček, V., Radojčić Redovniković, I. (2015a) Cholinium-based deep eutectic solvents and ionic liquids for lipase-catalyzed synthesis of butyl acetate. J. *Mol. Catal. B Enzym.* **122**, 188–198.

Cvjetko Bubalo, M., Mazur, M., Radošević, K., Radojčić Redovniković, I. (2015b) Baker's yeast-mediated asymmetric reduction of ethyl 3-oxobutanoate in deep eutectic solvents. *Process Biochem.* **50**, 1788–1792.

Cvjetko Bubalo, M., Panić, M., Radošević, K., Radojčić Redovniković,I. (2016a) Methods for deep eutectics solvents preparation. *Croatian Journal of Food Technology, Biotechnology and Nutrition* **11**, 164-168.

Cvjetko Bubalo, M., Vidovic, S., Radojcic Redovnikovic, I., Jokic, S. (2015c) Green solvents for green technologies. *J. Chem. Technol. Biotechnol.* **90**, 1631–1639.

Cvjetko Bubalo, M., Vidović, S., Radojčić Redovniković, I., Jokić, S. (2018) New perspective in extraction of plant biologically active compounds by green solvents. *Food Bioprod. Process.* **109**, 52–73.

Cvjetko Bubalo, M., Vidović, S., Radojčić Redovniković,I., Jokić, S. (2015) Green solvents for green technologies. *J. Chem. Technol. Biotechnol.* **90**, 1631–1639.

Dafoe, J.T., Daugulis, A.J. (2014) In situ product removal in fermentation systems: Improved process performance and rational extractant selection. *Biotechnol. Lett.* **36**, 443–460.

Dai, Y., Rozema, E., Verpoorte, R., Choi, Y.H. (2016) Application of natural deep eutectic solvents to the extraction of anthocyanins from Catharanthus roseus with high extractability and stability replacing conventional organic solvents. *J. Chromatogr. A* **1434**, 50–56.

Dai, Y., van Spronsen, J., Witkamp, G.J., Verpoorte, R., Choi, Y.H. (2013a) Natural deep eutectic solvents as new potential media for green technology. *Anal. Chim. Acta* **766**, 61–68.

Dai, Y., Van Spronsen, J., Witkamp, G.J., Verpoorte, R., Choi, Y.H. (2013b) Ionic liquids and deep eutectic solvents in natural products research: Mixtures of solids as extraction solvents. *J. Nat. Prod.* **76**, 2162–2173.

Dai, Y., Verpoorte, R., Choi, Y.H. (2014) Natural deep eutectic solvents providing enhanced stability of natural colorants from safflower (Carthamus tinctorius). *Food Chem.* **159**, 116–121. 5

Dai, Y., Witkamp, G.J., Verpoorte, R., Choi, Y.H. (2013c) Natural deep eutectic solvents as a new extraction media for phenolic metabolites in carthamus tinctorius L. *Anal. Chem.* **85**, 6272–6278.

Dai, Y., Witkamp, G.J., Verpoorte, R., Choi, Y.H. (2015) Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. *Food Chem.* **187**, 14-19.

Durand, E., Lecomte, J., Baréa, B., Piombo, G., Dubreucq, E., Villeneuve, P. (2012) Evaluation of deep eutectic solvents as new media for Candida antarctica B lipase catalyzed reactions. *Process Biochem.* **47**, 2081–2089.

Durand, E., Lecomte, J., Upasani, R., Chabi, B., Bayrasy, C., Baréa, B., Jublanc, E., Clarke, M.J., Moore, D.J., Crowther, J., Wrutniak-Cabello, C.,Villeneuve, P. (2017) Evaluation of the ROS Inhibiting Activity and Mitochondrial Targeting of Phenolic Compounds in Fibroblast Cells Model System and Enhancement of Eff iciency by Natural Deep Eutectic Solvent (NADES) Formulation. *Pharm. Res.* **34**, 1134-1146.

Dziedzic, A., Kubina, R., Kabała-Dzik, A., & Tanasiewicz, M. (2017) Induction of Cell Cycle Arrest and Apoptotic Response of Head and Neck Squamous Carcinoma Cells (Detroit 562) by Caffeic Acid and Caffeic Acid Phenethyl Ester Derivative. *Evid. Based Complement Alternat. Med.* ID 6793456

Eckert, F., Klamt, A. (2002) Fast Solvent Screening via Quantum Chemistry: COSMO-RS Approach. *AIChE J.* **48**, 369–385.

El Kantar, S., Rajha, H.N., Boussetta, N., Vorobiev, E., Maroun, R.G., Louka, N. (2019) Green extraction of polyphenols from grapefruit peels using high voltage electrical discharges, deep eutectic solvents and aqueous glycerol. *Food Chem.* **295**, 165–171.

Elgharbawy, A.A. (2018) Shedding Light on Lipase Stability in Natural Deep Eutectic Solvents. *Chem. Biochem. Eng. Q.* **32**, 359–370.

Faggian, M., Sut, S., Perissutti, B., Baldan, V., Grabnar, I., Dall'Acqua, S. (2016) Natural Deep Eutectic Solvents (NADES) as a tool for bioavailability improvement: Pharmacokinetics of rutin dissolved in proline/glycine after oral administration in rats: Possible application in nutraceuticals. *Molecules* **21**, 1–11.

Fernández, M.D.L.Á., Espino, M., Gomez, F.J. V, Silva, M.F. (2018) Novel approaches mediated by tailor-made green solvents for the extraction of phenolic compounds from agro-food industrial by-products. *Food Chem.* **239**, 671–678.

Florindo, C., Oliveira, F.S., Rebelo, L.P.N., Fernandes, A.M., Marrucho, I.M. (2014) Insights into the synthesis and properties of deep eutectic solvents based on choline chloride and carboxylic acids. *ACS Sustain. Chem. Eng.* **2**, 2416–2425.

Francisco, M., Van Den Bruinhorst, A., Kroon, M.C. (2013) Low-transition-temperature mixtures (LTTMs): A new generation of designer solvents. *Angew. Chemie - Int. Ed.* **52**, 3074–3085.

Gali-Muhtasib, H., Hmadi, R., Kareh, M., Tohme, R., Darwiche, N. (2015) Cell death mechanisms of plant-derived anticancer drugs: beyond apoptosis. *Apoptosis* **20**, 1531-1562.

Giusti, M.M., Sigurdson, G.T. (2019) Advances in Anthocyanin Research 2018, Molecules.

González, C.G., Mustafa, N.R., Wilson, E.G., Verpoorte, R., Choi, Y.H. (2018) Application of natural deep eutectic solvents for the "green"extraction of vanillin from vanilla pods. *Flavour Fragr. J.* **33**, 91–96.

Gorke, J., Srienc, F., Kazlauskas, R. (2010) Toward advanced ionic liquids. Polar, enzyme-friendly solvents for biocatalysis. *Biotechnol. Bioprocess Eng.* **15**, 40–53.

Guajardo, N., Ahumada, K., Domínguez de María, P., Schrebler, R.A. (2019) Remarkable stability of Candida antarctica lipase B immobilized via cross-linking aggregates (CLEA) in deep eutectic solvents. *Biocatal. Biotransfor.* **37**, 106–114.

Guajardo, N., Domínguez de María, H.P., Ahumada, K., Schrebler, R.A., Ramírez-Tagle, R., Crespo, F.A., Carlesi, C. (2017) Water as Cosolvent: Nonviscous Deep Eutectic Solvents for Efficient Lipase-Catalyzed Esterifications. *ChemCatChem* **9**, 1393–1396.

Gutiérrez, M.C., Carriazo, D., Ania, C.O., Parra, J.B., Ferrer, M.L., Del Monte, F. (2011) Deep eutectic solvents as both precursors and structure directing agents in the synthesis of nitrogen doped hierarchical carbons highly suitable for CO_2 capture. *Energy Environ. Sci.* **4**, 3535-3544

Harguindey, S., Stanciu, D., Devesa, J., Alfarouk, K., Cardone, R. A., Polo Orozco, J. D., Devesa, P., Rauch, C., Orive, G., Anitua, E., Roger, S., & Reshkin, S. J. (2017) Cellular acidification as a new approach to cancer treatment and to the understanding and therapeutics of neurodegenerative diseases. *Semin. Cancer Biol.* **43**, 157-179.

Hayyan, M., Hashim, M.A., Al-Saadi, M.A., Hayyan, A., AlNashef, I.M., Mirghani, M.E.S. (2013) Assessment of cytotoxicity and toxicity for phosphonium-based deep eutectic solvents. *Chemosphere* **93**, 455–459.

Huang, Y., Feng, F., Jiang, J., Qiao, Y., Wu, T., Voglmeir, J., Chen, Z.-G. (2016) Green and efficient extraction of rutin from tartary buckwheat hull by using natural deep eutectic solvents. *Food Chem.* **221**, 1400–1405.

Huang, Z.L., Wu, B.P., Wen, Q., Yang, T.X., Yang, Z. (2014) Deep eutectic solvents can be viable enzyme activators and stabilizers. *J. Chem. Technol. Biotechnol.* **89**, 1975–1981.

Jeong, K.M., Ko, J., Zhao, J., Jin, Y., Yoo, D.E., Han, S.Y., Lee, J. (2017) Multi-functioning deep eutectic solvents as extraction and storage media for bioactive natural products that are readily applicable to cosmetic products. *J. Clean. Prod.* **151**, 87–95.

Jeong, K.M., Lee, M.S., Nam, M.W., Zhao, J., Jin, Y., Lee, D.K., Kwon, S.W., Jeong, J.H., Lee, J. (2015a) Tailoring and recycling of deep eutectic solvents as sustainable and efficient extraction media. *J. Chromatogr. A* **1424**, 10–17.

Jeong, K.M., Zhao, J., Jin, Y., Heo, S.R., Han, S.Y., Yoo, D.E., Lee, J. (2015b) Highly efficient extraction of anthocyanins from grape skin using deep eutectic solvents as green and tunable media. *Arch. Pharm. Res.* **38**, 2143–2152.

Jiang, H., Diao, B., Xu, D., Zhang, L., Ma, Y., Gao, J., Wang, Y. (2019) Deep eutectic solvents effect on vapor-liquid phase equilibrium for separation of allyl alcohol from its aqueous solution. *J. Mol. Liq.* **279**, 524–529.

Juneidi, I., Hayyan, M., Hashim, M.A., Hayyan, A. (2017) Pure and aqueous deep eutectic solvents for a lipase-catalysed hydrolysis reaction. *Biochem. Eng. J.* **117**, 129–138.

Juneidi, I., Hayyan, M., Mohd Ali, O. (2016) Toxicity profile of choline chloride-based deep eutectic solvents for fungi and Cyprinus carpio fish. *Environ. Sci. Pollut. Res.* **23**, 7648–7659.

Khandelwal, S., Tailor, Y.K., Kumar, M. (2016) Deep eutectic solvents (DESs) as eco-friendly and sustainable solvent/catalyst systems in organic transformations. *J. Mol. Liq.* **215**, 345–386.

Lakka, A., Grigorakis, S., Karageorgou, I., Batra, G., Kaltsa, O., Bozinou, E., Lalas, S., Makris, D.P. (2019) Saffron processing wastes as a bioresource of high-value added compounds: Development of a green extraction process for polyphenol recovery using a natural deep eutectic solvent. *Antioxidants* **8**, 586.

Leonelli, C., Mason, T.J. (2010) Chemical Engineering and Processing : Process Intensification Microwave and ultrasonic processing : Now a realistic option for industry. *Chem. Eng. Process. Process Intensif.* **49**, 885–900.

Liang, F., Huang, J., He, J., Wang, P. (2012) Improved Enantioselective Hydrolysis of Racemic Ethyl-2 , 2- dimethylcyclopropanecarboxylate Catalyzed by Modified Novozyme 435. *Biotechnol. Bioproc. Eng.* **958**, 952–958.

Liu, X., Ahlgren, S., Korthout, H.A.A.J., Salomé-Abarca, L.F., Bayona, L.M., Verpoorte, R., Choi, Y.H. (2018) Broad range chemical profiling of natural deep eutectic solvent extracts using a high performance thin layer chromatography–based method. *J. Chromatogr. A* **1532**, 198–207.

Liu, Y., Friesen, J.B., McAlpine, J.B., Lankin, D.C., Chen, S.N., Pauli, G.F. (2018) Natural Deep Eutectic Solvents: Properties, Applications, and Perspectives. *J. Nat. Prod.* **81**, 679–690.

Liu, Y., Garzon, J., Friesen, J.B., Zhang, Y., McAlpine, J.B., Lankin, D.C., Chen, S.N., Pauli, G.F. (2016) Countercurrent assisted quantitative recovery of metabolites from plant-associated natural deep eutectic solvents. *Fitoterapia* **112**, 30–37.

Liu, Z.H., Luo, Z.W., Li, D.W., Wang, D.M. & Ji, X. (2018) Synergistic effects and related bioactive mechanisms of Potentilla fruticosa Linn. leaves combined with green tea polyphenols studied with microbial test system (MTS). *Nat. Pro. Res.* **32**, 1287-1290.

Makris, D.P. (2018) Green extraction processes for the efficient recovery of bioactive polyphenols from wine industry solid wastes – Recent progress. *Curr. Opin. Green Sustain. Chem.* **13**, 50–55.

Mao, S., Li, K., Hou, Y., Liu, Y., Ji, S., Qin, H., Lu, F. (2018) Synergistic effects of components in deep eutectic solvents relieve toxicity and improve the performance of steroid biotransformation catalyzed by Arthrobacter simplex. *J. Chem. Technol. Biotechnol.* **93**, 2729–2736.

Mao, S., Wang, K., Lei, Y., Yao, S., Lu, B. & Huang, W. (2017) Antioxidant synergistic effects of Osmanthus fragrans flowers with green tea and their major contributed antioxidant compounds. *Sci. Rep.* **7**, 46501-46511.

Mao, S., Yu, L., Ji, S., Lu, F. (2015) Evaluation of deep eutectic solvents as co-solvent for steroids 1-en-dehydrogenation biotransformation by *Arthrobacter simplex*. J. *Chem. Technol. Biotecnol.* **91**, 1099-1104.

Martins, M.A.R., Pinho, S.P., Coutinho, J.A.P. (2019) Insights into the Nature of Eutectic and Deep Eutectic Mixtures. *J. Solution Chem.* **48**, 962–982.

Maugeri, Z., Domínguez De María, P. (2014) Whole-cell biocatalysis in deep-eutectic-solvents/aqueous mixtures. *ChemCatChem* **6**, 1535–1537.

Maugeri, Z., Leitner, W., Domínguez De María, P. (2012) Practical separation of alcohol-ester mixtures using Deep-Eutectic-Solvents. *Tetrahedron Lett.* **53**, 6968–6971.

Mbous, Y.P., Hayyan, M., Hayyan, A., Wong, W.F., Hashim, M.A., Looi, C.Y. (2017a) Applications of deep eutectic solvents in biotechnology and bioengineering: Promises and challenges. *Biotechnol. Adv.* **35**, 105–134.

Mbous, Y.P., Hayyan, M., Wong, W.F., Looi, C.Y., Hashim, M.A. (2017b) Unraveling the cytotoxicity and metabolic pathways of binary natural deep eutectic solvent systems. *Sci. Rep.* **7**, 41257.

Mitar, A., Panić, M., Kardum, J.P., Halambek, J., Sander, A. (2019) Physicochemical Properties, Cytotoxicity, and Antioxidative Activity of Natural Deep Eutectic Solvents Containing Organic Acid. *Chem. Biochem. Eng. Q*. **33**, 39–56.

Mitar, A., Prlić Kardum, J., Sander, A., Blažanović, M. (2018) Deep eutectic solvents as extraction media for fuel purification. *Procceding of Natural Resources, Green Technology & Sustainable Development* **3**, 35-40.

Mohamad Hamdi Zainal Abidin, Maan Hayyan, Adeeb Hayyan, J.S.N. (2017) New Horizons in the Extraction of Bioactive Compounds using. *Anal Chim Acta.* **979**, 1-23.

Monhemi, H., Housaindokht, M.R., Moosavi-Movahedi, A.A., Bozorgmehr, M.R. (2014) How a protein can remain stable in a solvent with high content of urea: Insights from molecular dynamics simulation of Candida antarctica lipase B in urea: Choline chloride deep eutectic solvent. *Phys. Chem. Chem. Phys.* **16**, 14882–14893.

Mouratoglou, E., Malliou, V., Makris, D.P. (2016) Novel Glycerol-Based Natural Eutectic Mixtures and Their Efficiency in the Ultrasound-Assisted Extraction of Antioxidant Polyphenols from Agri-Food Waste Biomass. *Waste Biomass Valori.* **7**, 1377–1387.

Müller, C.R., Lavandera, I., Gotor-Fernández, V., Domínguez de María, P. (2015) Performance of recombinant-whole-cell-catalyzed reductions in deep-eutectic-solvent-aqueous-media mixtures. *ChemCatChem* **7**, 2654–2659.

Nam, M.W., Zhao, J., Lee, M.S., Jeong, J.H., Lee, J. (2015) Enhanced extraction of bioactive natural products using tailor-made deep eutectic solvents: application to flavonoid extraction from Flos sophorae. *Green Chem.* **17**, 1718–1727.

Nguyen, L.A., He, H., Pham-Huy, C. (2006) Chiral drugs: an overview. *Int. J. Biomed. Sci.* **2**, 85–100.

Ogihara, W., Aoyama, T., Ohno, H. (2004) Polarity Measurement for Ionic Liquids Containing Dissociable Protons. *Chem. Lett.* **33**, 1414–1415.

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Reis, R.L., Duarte, A.R.C. (2014) Natural deep eutectic solvents - Solvents for the 21st century. *ACS Sustain. Chem. Eng.* **2**, 1063–1071.

Paiva, A., Matias, A.A., Duarte, A.R.C. (2018) ScienceDirect How do we drive deep eutectic systems towards an industrial reality? *Curr. Opin. Green Sustain. Chem.* **11**, 81–85.

Panić, M., Delač, D., Roje, M., Radojčić Redovniković, I., Cvjetko Bubalo, M. (2018) Green asymmetric reduction of acetophenone derivatives : *Saccharomyces cerevisiae* and aqueous natural deep eutectic solvent. *Biotechnol Lett.* **41**, 253-262.

Panić, M., Gunjević, V., Cravotto, G., Radojčić Redovniković, I. (2019a) Enabling technologies for the extraction of grape-pomace anthocyanins using natural deep eutectic solvents in up-to-half-litre batches. *Food Chem.* **300**, 125185.

Panić, M., Majerić Elenkov, M., Roje, M., Cvjetko Bubalo, M., Radojčić Redovniković, I. (2017) Plant-mediated stereoselective biotransformations in natural deep eutectic solvents. *Process Biochem.* **66**, 133-139.

Panić, M., Stojković, M.R., Kraljić, K., Škevin, D. (2019b) Ready-to-use green polyphenolic extracts from food by-products. *Food Chem.* **283**, 628–636.

Park, S., Shin, H., Cho, Y. (2013) Shikonin induces programmed necrosis-like cell death through the formation of receptor interacting protein 1 and 3 complex. *Food Chem. Toxicol.* **55**, 36-41.

Pätzold, M., Weimer, A., Liese, A., Holtmann, D. (2019) Optimization of solvent-free enzymatic esterification in eutectic substrate reaction mixture. *Biotechnol. Reports* **22**, e00333.

Pätzold, Magdalena, Siebenhaller, S., Kara, S., Liese, A., Syldatk, C., Holtmann, D. (2019) Deep Eutectic Solvents as Efficient Solvents in Biocatalysis. *Trends Biotechnol.* **9**, 1–17.

Pöhnlein, M., Ulrich, J., Kirschhöfer, F., Nusser, M., Muhle-Goll, C., Kannengiesser, B., Brenner-Weiß, G., Luy, B., Liese, A., Syldatk, C., Hausmann, R. (2015) Lipase-catalyzed synthesis of glucose-6-O-hexanoate in deep eutectic solvents. *Eur. J. Lipid Sci. Technol.* **117**, 161–166.

Radošević, K., Cvjetko Bubalo, M., Gaurina Srček, V., Grgas, D., Landeka Dragičević, T., Radojčić Redovniković, I. (2015) Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. *Ecotoxicol. Environ. Saf.* **112**, 46–53.

Radošević, K., Cvjetko Bubalo, M., Slivac, I., Gaurina Srček, V., Radojčić Redovniković, I. (2016a) Green technology meets ecotoxicology. Croat. *J. Food Sci. Technol.* **8**, 120–128.

Radošević, K., Čanak, I., Panić, M. Markov, K., Cvjetko Bbalo, M., Frece, J., Gaurina Srček, V., Radojčić Redovniković, I. (2016b) Antimicrobial, cytotoxic and antioxidative evaluation of natural deep eutectic solvents. *Environ. Sci. Pollut. Res.* **25***,* 14188-14196.

Radošević, K., Ćurko, N., Gaurina Srček, V., Cvjetko Bubalo, M., Tomašević, M., Kovačević Ganić, K., Radojčić Redovniković, I. (2016c) Natural deep eutectic solvents as beneficial extractants for enhancement of plant extracts bioactivity. *LWT - Food Sci. Technol.* **73**, 45–51.

Ranganathan, S., Zeitlhofer, S., Sieber, V. (2017) Development of a lipase-mediated epoxidation process for monoterpenes in choline chloride-based deep eutectic solvents. *Green Chem.* **19**, 2576–2586.

Reichardt, C. (1994) Solvatochromic Dyes as Solvent Polarity Indicators. *Chem. Rev.* **94**, 2319–2358.

Ribéreau-Gayon, P., Stonestreet, E. (1966) Dosage des tanins du vin rouge et détermination de leur structure. *Chim. Anal. Paris.* **48**, 188-196.

Rios, N.S., Pinheiro, M.P., Cleiton, J., Santos, S., Fonseca, T.D.S., Lima, L.D., Mattos, M.C. De, Freire, D.M.G., Ivanildo, J., Júnior, S., Rodríguez-aguado, E., Gonc, L.R.B. (2016) Enzymatic Strategies of covalent immobilization of a recombinant Candida antarctica lipase B on pore-expanded SBA-15 and its application in the kinetic resolution of (*R*, *S*) -Phenylethyl acetate. *J. Mol. Catal. B* **133**, 246–258.

Ruesgas-Ramon, M., Figueroa-Espinoza, M.C., Durand, E. (2017) Application of Deep Eutectic Solvents (DES) for phenolic compounds extraction: overview, challenges, and opportunities. *J. Agric. Food Chem.* **65**, 3591–3601.

Ruesgas-Ramón, M., Suárez-Quiroz, M.L., González-Ríos, O., Baréa, B., Cazals, G., Figueroa-Espinoza, M.C., Durand, E. (2019) Biomolecules extraction from coffee and cocoa by- and co-products using deep eutectic solvents. *J. Sci. Food Agric*. **100**, 81-91.

Schieber, M., & Chandel, N. S. (2014) ROS function in redox signaling and oxidative stress. *Curr. Biol.*, **24**, 453-462.

Shahidi, F. & Ambigaipalan, P. (2015) Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects – A review. *J. Funct. Foods* **18**, 820-897.

Sheldon, R.A. (2016) The E factor 25 years on: the rise of green chemistry and sustainability. *Green Chem.* **19**, 18–43.

Siebenhaller, S., Muhle-Goll, C., Luy, B., Kirschhöfer, F., Brenner-Weiss, G., Hiller, E., Günther, M., Rupp, S., Zibek, S., Syldatk, C. (2016) Sustainable enzymatic synthesis of glycolipids in a deep eutectic solvent system. *J. Mol. Catal. B Enzym.* **133**, S281–S287.

Silva, J.M., Reis, R.L., Paiva, A., Duarte, A.R.C. (2018) Design of Functional Therapeutic Deep Eutectic Solvents Based on Choline Chloride and Ascorbic Acid. *ACS Sustain. Chem. Eng.* **6**, 10355-10363.

Singleton, V. L., Orthofer, R., Lamuela-Raventos, R. M. (1999) Analysis of total phenols and other oxidation substrates and antioxidants means of Folin Ciocalteu reagent. *Method. Enzymol.*, **299**,152-178.

Sontakke, J.B., Yadav, G.D. (2011) Optimization and kinetic modeling of lipase catalyzed enantioselective N-acetylation of (±)-1-phenylethylamine under microwave irradiation. *J. Chem. Technol. Biotechnol.* **86**, 739–748.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., Garcia-Viguera, C. (2014) Natural bioactive compounds from winery by-products as health promoters: A review. *Int. J. Mol. Sci.* **15**, 15638–15678.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., & Garcia-Viguera, C. (2014) Natural bioactive compounds from winery by-products as health promoters: A review. *Int. J. Mol. Sci.* **15**, 15638–15678.

U.S. Department of Health and Human Services, National Institutes of Health, Office of Dietary Supplements (2018) Choline: Fact Sheet for Health Professionals. Retrieved from https://ods.od.nih.gov/factsheets/Choline-HealthProfessional/#disc

Ülger, C., Takaç, S. (2017) Kinetics of lipase-catalysed methyl gallate production in the presence of deep eutectic solvent. *Biocatal. Biotransformation* **35**, 407–416.

Vanda, H., Dai, Y., Wilson, E.G., Verpoorte, R., Hae, Y. (2018) Green solvents from ionic liquids and deep eutectic solvents to natural deep eutectic solvents. *Comptes rendus - Chim.* **21**, 1–11.

Wang, T., Jiao, J., Gai, Q.-Y., Wang, P., Guo, N., Niu, L.-L., Fu, Y.-J. (2017) Enhanced and green extraction polyphenols and furanocoumarins from Fig (*Ficus carica* L.) leaves using deep eutectic solvents. *J. Pharm. Biomed. Anal.* **145**, 339–345.

Wei, P., Liang, J., Cheng, J., Zong, M.-H., Lou, W.-Y. (2016) Markedly improving asymmetric oxidation of 1-(4-methoxyphenyl) ethanol with Acetobacter sp. CCTCC M209061 cells by adding deep eutectic solvent in a two-phase system. *Microb. Cell Fact.* **15**, 5.

Wen, Q., Chen, J.X., Tang, Y.L., Wang, J., Yang, Z. (2015) Assessing the toxicity and biodegradability of deep eutectic solvents. *Chemosphere* **132**, 63–69.

Wohlgemuth, R. (2017) Biocatalytic Process Design and Reaction Engineering. *Chem. Biochem. Eng. Q.* **31**, 131–138.

Woodley, J.M. (2008) New opportunities for biocatalysis: making pharmaceutical processes greener. *Trends Biotechnol.* **26**, 321–327.

Xia, E., He, X., Li, H., Wu, S., Li, S., & Deng, G. (2010) Biological Activities of Polyphenols from Grapes. *Int.J. Mol. Sci.* **11**,622-646.

Xie, P., Zhou, X., Zheng, L. (2019) Stereoselective synthesis of a key chiral intermediate of (*S*)-Rivastigmine by AKR-GDH recombinant whole cells. *J. Biotechnol.* **289**, 64–70.

Xu, P., Du, P.-X., Zong, M.-H., Li, N., Lou, W.-Y. (2016) Combination of deep eutectic solvent and ionic liquid to improve biocatalytic reduction of 2-octanone with *Acetobacter pasteurianus* GIM1.158 cell. *Sci. Rep.* **6**, 26158.

Xu, P., Xu, Y., Li, X.F., Zhao, B.Y., Zong, M.H., Lou, W.Y. (2015) Enhancing asymmetric reduction of 3-chloropropiophenone with immobilized acetobacter sp. CCTCC M209061 cells by using deep eutectic solvents as cosolvents. *ACS Sustain. Chem. Eng.* **3**, 718–724.

Yang, T.X., Zhao, L.Q., Wang, J., Song, G.L., Liu, H.M., Cheng, H., Yang, Z. (2017) Improving Whole-Cell Biocatalysis by Addition of Deep Eutectic Solvents and Natural Deep Eutectic Solvents. *ACS Sustain. Chem. Eng.* **5**, 5713–5722.

Yang, Z. (2018) Natural Deep Eutectic Solvents and Their Applications in Biotechnology. In: Application of Ionic Liquids in Biotechnology, Springer, pp 31-59.

Yoshikawa, K., Saito, S., & Sakuragawa, A. (2011) Simultaneous analysis of acidulants and preservatives in food samples by using capillary zone electrophoresis with indirect UV detection. *Food Chem.* **127**, 1385–1390.

Zanke, B. W., Lee, C., Arab, S., & Tannock, I.F. (1998) Death of Tumor Cells after Intracellular Acidification Is Dependent on Stress-activated Protein Kinases (SAPK/JNK) Pathway Activation and Cannot Be Inhibited by Bcl-2 Expression or Interleukin 1β-converting Enzyme Inhibition. *Cancer Research* **5**, 2801-2808.

Zeng, C.X., Qi, S.J., Xin, R.P., Yang, B., Wang, Y.H. (2015) Enzymatic selective synthesis of 1,3-DAG based on deep eutectic solvent acting as substrate and solvent. *Bioprocess Biosyst. Eng.* **38**, 2053–2061.

Zhang, H., Tang, B., Row, K. (2014) Extraction of catechin compounds from green tea with a new green solvent. *Chem. Res. Chinese Univ.* **30**, 37–41.

Zhao, B.Y., Xu, P., Yang, F.X., Wu, H., Zong, M.H., Lou, W.Y. (2015) Biocompatible Deep Eutectic Solvents Based on Choline Chloride: Characterization and Application to the Extraction of Rutin from *Sophora japonica*. ACS Sustain. *Chem. Eng.* **3**, 2746-2755

Zhou, P., Wang, X., Liu, P., Huang, J., Wang, C., Pan, M., Kuang, Z. (2018) Enhanced phenolic compounds extraction from Morus alba L. leaves by deep eutectic solvents combined with ultrasonic-assisted extraction. *Ind. Crops Prod.* **120**, 147–154.

Zhuang, B., Dou, L.L., Li, P., Liu, E.H. (2017) Deep eutectic solvents as green media for extraction of flavonoid glycosides and aglycones from Platycladi Cacumen. *J. Pharm. Biomed. Anal.* **134**, 214–219.

8. SUPPLEMENTS

8.1. List of symbols

Abbreviations

7-AAD	7-aminoactinomycin D
ANOVA	analysis of variance
BCit	Betaine:citric acid
BMa	Betaine:malic acid
ChCit	Choline chloride:citric acid
ChCl	Choline chloride
ChEG	Choline chloride:ethylene glycol
ChGlc	Choline chloride:glucose
ChGly	Choline chloride:glycerol
ChMa	Choline chloride:malic acid
ChProMa	Choline chloride:proline:malic acid
DAD	diode array detector
DHE	dihydroethidium
DW	dry weight
EGGlcFru	Ethylene glycol:glucose:fructose
GCMS	Gas chromatograph with mass spectrometry
GlcEG	Glucose:ethylene glycol
GlcGly	Glucose:glycerol
GPCCit	grape pomace extract in NADES ChCit (70%, v/v)
GPEtOH	grape pomace extract in aqueous ethanol (70%, v/v)
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HPLC	High-performance liquid chromatography
MAE	Microwave assisted extraction
MaGlc	Malic acid:glucose
MaGlcGly	Malic acid:glucose:glycerol
MW	microwave irradiation
NADES	Natural deep eutectic solvents
ORAC	Oxygen radical absorbance capacity

ProMa	Proline:malic acid
PTFE	polytetrafluoroethylene
RSM	Response surface methodology
SD	standard deviation
SorEG	Sorbose:ethylene glycol
TA	Total anthocyanin content
TP	Total phenolic content
UAE	Ultrasound assisted extraction
UMAE	simultaneous ultrasound/microwave-assisted extraction
US	Ultrasound irradiation

Parameters and kinetic constants

A	residual activity	%
ee	enantiomeric excess	%
E_{NR}	molar transition energy	kcal mol^{-1}
k_o	Initial reaction rate	mmoL L^{-1} min^{-1}
N_A	Avogadro's constant	mol^{-1}
P	power	W
Relative ORAC value		μmol TE g^{-1}
V_p	volumetric productivity	mmol L^{-1} min^{-1}
$V_{relative}$	NADES reusability	%
X	reaction conversion	%
$\eta_{relative}$	enzyme reusability	%
λ_{max}	maximum absorption wavelength	nm
L	energy consumption	kW h
w	content	mg g^{-1}
η	separation efficiency	%

Curriculum vitae

Manuela Panić was born in Čakovec, 27th October 1991. She graduated from the Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia, where she obtained title Master of bioprocess engineering in 2016. Her Master thesis was done in the Laboratory for Cell Technology, Application and Biotransformations under the supervision of Associate Professor Kristina Radošević. The same year she enrolled in a postgraduate Ph.D. study in *Biotechnology and Bioprocess Engineering, Food Technology and Nutrition* at the same Faculty.

Since 2016 she has been working as a research assistant at the same Faculty in the Laboratory for Cell Technology, Application and Biotransformations, on a scientific project "*Green solvents for green technologies*" (project number: 9550) led by Professor Ivana Radojčić Redovniković. During her Ph.D, she was on traineeship for 7 months at the University of Turin. In 2019 she was working on Precious project in Italy in collaboration with the cosmetic and food industry, where she prepared grape pomace extracts stabilized on mesoporous supports and cyclodextrins. Since 2020 she is a team member on the project „*Rational design of natural deep eutectic solvents for chiral drugs preparation and formulation*" founded by Croatian science foundation and on the bilateral Chinese-Croatian and Austrian-Croatian scientific project.

She is involved in teaching activities as assistance in following courses for students: *HPLC of low molecular compound*, *Biotransformation* and *Phytoremediation*. Her work has been acknowledged with several awards: Biotechnical foundation scholarship (2017), Scholarship by British Embassy Zagreb for participating on *Bioprocess Engineering Course* (2018), Faculty of Food Technology and Biotechnology award for young scientist (2019), University of Turin Scholarship (2019), Annual Award of the Society for Young Scientists and Artists (2020).

She was a Member of the organization committee of *Natural resources, green technology and sustainable development-GREEN3*. She published 7 a1 papers, 1 a2 paper, 1 a3 and 3 professional papers. She participated in 17 international and 4 national conferences. She also popularized science in 2017 by participating in a University of Zagreb show entitled *Green Solvents for Green Technologies*.

Životopis

Manuela Panić rođena je 27. listopada 1991.g. u Čakovcu. Godine 2016. diplomirala je Bioprocesno inženjerstvo na Prehrambeno-biotehnološkom fakultetu, Sveučilišta u Zagrebu s izvrsnim uspjehom, a diplomski rad izradila je u Laboratoriju za tehnologiju i primjenu stanica i biotransformacije pod mentorstvom izv.prof.dr.sc. Kristine Radošević. Iste je godine upisala poslijediplomski studij *Biotehnologija i bioprocesno inženjerstvo, prehrambena tehnologija i nutricionizam* na Prehrambeno-biotehnološkom fakultetu.

Od 3. studenoga 2016. g. zaposlena je kao asistent u Laboratoriju za tehnologiju i primjenu stanica i biotransformacije na projektu *Zelena otapala za zelene tehnologije* (9550) pod vodstvom prof.dr.sc. Ivane Radojčić Redovniković. Tijekom poslijediplomskog studija bila je na stručnom usavršavanju na Sveučilištu u Torinu, Italija, u trajanju od 7 mjeseci. Godine 2019. radila je kao suradnik na projektu Precious (Sveučilište u Torinu, Italija), gdje je bila odgovorna za pripremu ekstrakata komine grožđa stabiliziranima na mezoporoznim nosačima i ciklodekstrinima za industrijsku primjenu. Od 2020. godine suradnica je na projektu *Racionalan dizajn prirodnih eutektičkih otapala za pripremu i formulaciju kiralnih lijekova* Hrvatske zaklade za znanost, te bilateralnom znanstveno-istraživačkom projektu s Narodnom Republikom Kinom (Sveučilište Anhui) i Austrijom (Sveučilište u Grazu).

Suradnica je na kolegijima *HPLC niskomolekulskih spojeva*, *Biotransformacije* i *Fitoremedijacija*. Godine 2017. nagrađena je za svoj znanstveno-istraživački rad s nekoliko nagrada: Potporom Biotehničke zaklade (2017.), Stipendijom British Embassy Zagreb za sudjelovanje na *Bioprocess Engineering Course 2018* (2018.), Nagradom za uspješnog mladog znanstvenika Prehrambeno-biotehnološkog fakulteta (2019.), Stipendijom Sveučilita u Torinu (2019.) te Godišnjom nagradom Društva sveučilišnih nastavnika i drugih znanstvenika mladim znanstvenicima i umjetnicima (2020.).

Manuela Panić bila je i član organizacijskog i uređivačkog odbora Međunarodne konferencije *Natural resources, green technology and sustainable development 3*. Do sada je objavila 7 znanstvenih a1 radova, 1 a2, 1 a3 te 3 stručna rada. Sudjelovala je na 17 međunarodnih 4 domaća znanstvena skupova. Godine 2017. sudelovanjem u emisiji Sveučilišnog Zagreba pod naslovom *Zelena otapala za zelene tehnologije*.

Curriculum vitae

Published papers:

a1

1. Radošević, K., Čanak, I., **Panić, M**., Markov, K., Cvjetko Bubalo, M., Frece, J., Radojčić Redovniković, I. (2018) Antimicrobial, cytotoxic and antioxidative evaluation of natural deep eutectic solvents. *Environ. Sci. Pollut. Res.* **25**, 14188-14196.
2. Valinger, D., Kušen, M., Jurinjak Tušek, A., **Panić, M.,** Jurina, T., Benković, M., Radojčić Redovniković, I., Gajdoš Kljusurić, J. (2018) Development of Near Infrared Spectroscopy Models for the Quantitative Prediction of Olive Leaves Bioactive Compounds. *Chem. Biochem. Eng. Q.* **32**, 535-543.
3. **Panić, M.**, Majerić Elenkov, M., Roje, M., Cvjetko Bubalo, M., Radojčić Redovniković, I. (2018) Plant-mediated stereoselective biotransformations in natural deep eutectic solvents. *Process Biochem.* **66**, 133-139.
4. **Panić, M.**, Delač, D., Roje, M., Radojčić Redovniković, I., Cvjetko Bubalo, M. (2019) Green asymmetric reduction of acetophenone derivatives: *Saccharomyces cerevisiae* and aqueous natural deep eutectic solvent. *Biotechnol. Lett.* **41**, 253-262.
5. **Panić, M.**, Radić Stojković, M., Kraljić, K., Škevin, D., Radojčić Redovniković, I., Gaurina Srček, V., Radošević, K. (2019) Ready-to-use green polyphenolic extracts from food by-products. *Food Chem.* **283**, 628-636.
6. **Panić, M.**, Gunjević, V., Cravotto, G., Radojčić Redovniković, I. (2019) Enabling technologies for the extraction of grape-pomace anthocyanins using natural deep eutectic solvents in up-to-half-litre batches. *Food Chem.* **300**, 125185.
7. Mitar, A., **Panić, M.**, Prlić Kardum, J. Halambek, J., Sander, A., Zagajski Kučan, K., Radojčić Redovniković, I., Radošević, K. (2019) Physicochemical Properties, Cytotoxicity, and Antioxidative Activity of Natural Deep Eutectic Solvents Containing Organic Acid, *Chem. Biochem. Eng. Q.* **33**, 39-56.
8. Miličević, N., **Panić, M.**, Valinger, D., Cvjetko Bubalo, M., Benković, M., Jurina, T., Gajdoš Kljusurić, J., Radojčić Redovniković, A., Jurinjak Tušek, A. (2020) Development of continuously operated aqueous two-phase microextraction process using natural deep eutectic solvents. *Sep. Purif. Technol.,* 116746. (*In press,* DOI: 10.1016/j.seppur.2020.116746)

a2

1. Jakovljević, T., **Panić, M.**, Cvjetko Bubalo, M., Čehulić, I., Radojčić Redovniković, I. (2017) Utjecaj kadmija na uzgoj kelja i kupusa u onečišćenim tlima. *Glasnik zaštite bilja*, **40**, 53-62.

a3

1. **Panić.**, **M.**, Košpić, K., Marin, P., Gukov, A., Šarac, A., Vukšić, M., Cvjetko Bubalo, M., Roje, M., Radojčić Redovniković, I., Environment-friendly biocatalytic processes using natural deep eutectic solvents. *Procceding of Natural Resources, Green Technology & Sustainable Development 3* (Radojčić Redovniković, Ivana ; Jakovljević, Tamara ; Petravić Tominac, Vlatka; Panić, Manuela ; Stojaković, Renata ; Erdec, Dina ; Radošević, Kristina ; Gaurina Sarček, Višnja ; Cvjetko Bubalo, Marina (ur.)). Zagreb: Prehrambeno-biotehnološki fakultet, 2018. str. 51-55.

Professional papers

1. Radojčić Redovniković, I., Cvjetko Bubalo, M., **Panić, M.**, Radošević, K. (2016) Biološki aktivni spojevi glukozinolati i polifenoli u cvatu, listu i stabljici brokule (*Brassica oleracea* var. italica L.). *Glasnik Zaštite Bilja*, **5**, 24-29. (ISSN 0350-9664)
2. Radojčić Redovniković, I., Cvjetko Bubalo, M., **Panić, M.**, Radošević, K. (2016) Uloga glukozinolata u obrani biljaka od nametnika. *Glasnik Zaštite Bilja*, **6**, 12-14. (ISSN 0350-9664)
3. Radojčić Redovniković, I., Cvjetko Bubalo, M., **Panić, M.**, Radošević, K., (2016) Metode priprave eutektičkih otapala. *Croatian Journal of Food Technology, Biotechnology and Nutrition* **11** (3-4), 164- 168. (ISSN 1847-3423)