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Development of green high performance solid-liquid extraction processes for the recovery of antioxidant polyphenols from the medicinal plant *Salvia fruticosa* Mill. (Cretan sage)

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Ανάπτυξη πράσινων διεργασιών εκχύλισης στερεού – υγρού υψηλής απόδοσης για την ανάκτηση πολυφαινολικών αντιοξειδωτικών από το φαρμακευτικό φυτό *Salvia fruticosa* Mill. (Κρητικό φασκόμηλο)

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Abstract

In the present work, the efficient extraction of polyphenols from the medicinal plant *S. fruticosa* Mill., was studied. For this purpose, environmentally benign solvents, additives and techniques were employed while optimization of extraction parameters secured the quantitative recovery of the biomolecules. The extractions performed were evaluated through antioxidant and kinetic assays.

In the first part, 3 cyclodextrins, namely β-cyclodextrin (β-CD), methyl-β-cyclodextrin (m-β-CD), and hydroxypropyl-β-cyclodextrin (HP-β-CD) were used as extraction booster of polyphenols from the *S. fruticosa* plant. The concentration of cyclodextrins (C_{CD}), the pH, and the liquid-to-solid ratio ($R_{l/s}$) were optimized by a Box-Behnken experimental design and kinetic study implemented to assess the effect of temperature on the extraction yield. According to the results, the m-β-CD was proved the most effective and the least energy demanding extraction booster. The phenolic profile analysis revealed that the major compounds of the phenolic fraction of the *S. fruticosa* species were Rosmarinic acid and Luteolin-7-O-glucuronide.

In the second part, the extraction capacity of a hydroglycerolic solution was tested and the effect of ultrasound pretreatment was also assessed, for the extraction of polyphenols from the *S. fruticosa* plant. The results showed that sonication pretreatment contributes to the overall extraction procedure and the time of the sonication affects, also, the phenolic yield. The combined extraction method (sonication + solvent extraction) exhibited comparable results to the previous cyclodextrin boosted extraction utilizing lower temperatures.

In the third part, novel deep eutectic solvents (DESs) were synthesized, the hydrogen bond donors (HBD) employed were glycerol (GL) and lactic acid (LA), while the hydrogen bond acceptors (HBAs) were citrate salts. After an initial screening, the most effective DES in terms of extraction capacity of *S. fruticosa* polyphenol, was the Lactic acid - Sodium citrate dibasic mixture, at molar ratio 15-1 (LA-SCDB15). The optimization of the extraction procedure with a Box-Behnken experimental design and the kinetic assay implemented, revealed that the DES presented higher extraction efficiency for the major salvia polyphenols and more energy efficient in comparison to the previously studied methods.

In the fourth and last part, the long-term and accelerated stability of the LA-SCDB15 extract were evaluated. As stability indexes, the antiradical activity (A_{AR}) and ferric reducing power (P_R), were used. The results of both assays showed that the DES extracts were stable under the tested conditions. The LC-DAD-MS analyses showed that the major compounds of the salvia species were unaffected by the stability study conditions employed.

Περίληψη

Στη παρούσα εργασία μελετήθηκε η αποτελεσματική εκχύλιση πολυφαινολών από το φαρμακευτικό φυτό *S.fruticosa*. Για αυτό το σκοπό χρησιμοποιήθηκαν περιβαλλοντικά, ήπιοι διαλύτες, πρόσθετα και τεχνικές, ταυτόχρονα, η βελτιστοποίηση των παραμέτρων εκχύλισης διασφάλισε την ποσοτική ανάκτηση των βιομορίων. Οι εκχυλίσεις που εφαρμόσθηκαν αξιολογήθηκαν με βάση αντιοξειδωτικές και κινητικές δοκιμασίες.

Στο πρώτο μέρος χρησιμοποιήθηκαν 3 κυκλοδεξτρίνες, συγκεκριμένα β-κυκλοδεξτρίνη (β-CD), μέθυλ-β-κυκλοδεξτρίνη, και υδρόξυ-πρόπυλ-β-κυκλοδεξτρίνη (HP-β-CD), χρησιμοποιήθηκαν ως ενισχυτικά εκχύλισης πολυφαινολών από το φυτό *S.fruticosa*. Η συγκέντρωση κυκλοδεξτρινών (C_{CD}), το pH, και η αναλογία υγρού προς στερεό ($R_{l/s}$) βελτιστοποιήθηκαν με τη χρήση πειραματικού σχεδιασμού κατά Box-Behnken και εφαρμόσθηκε κινητική μελέτη για να εκτιμηθεί η επίδραση της θερμοκρασίας στη απόδοση της εκχύλισης. Σύμφωνα με τα αποτελέσματα, η μέθυλ-β-κυκλοδεξτρίνη αποδείχθηκε το πιο αποτελεσματικό και λιγότερο ενεργοβόρο ενισχυτικό εκχύλισης. Η ανάλυση του φαινολικού προφίλ αποκάλυψε ότι οι βασικές ενώσεις του φαινολικού κλάσματος του γένους *S.fruticosa* ήταν το ροσμαρινικό οξύ και ο γλυκοζίτης της λουτεολίνης.

Στο δεύτερο μέρος, εξετάσθηκε η εκχυλιστική ικανότητα υδροαλκοολικού διαλύματος και η επίδραση της προεπεξεργασίας με υπέρηχους στην εκχύλιση πολυφαινολών από το φυτό *S.fruticosa*. Τα αποτελέσματα έδειξαν ότι η προεπεξεργασία με υπέρηχους συνεισφέρει στην συνολική διαδικασία εκχύλισης και ο χρόνος της επεξεργασίας με υπέρηχους, επίσης, επηρεάζει την απόδοση πολυφαινολών. Η συνδυασμένη μέθοδος εκχύλισης (επεξεργασία με υπέρηχους + εκχύλιση με διαλύτη) επέδειξε συγκρίσιμα αποτελέσματα με την προηγούμενη εκχύλιση με κυκλοδεξτρίνες χρησιμοποιώντας χαμηλότερες θερμοκρασίες.

Στο τρίτο μέρος, συνετέθησαν νέοι βαθέως ευτηκτικοί διαλύτες, οι δότες δεσμού υδρογόνου (HBD) που χρησιμοποιήθηκαν ήταν γλυκερόλη (GL) και γαλακτικό οξύ (LA), ενώ οι αποδέκτες δεσμού υδρογόνου (HBA) ήταν κιτρικά άλατα. Μετά από μια αρχική διαλογή, ο πιο αποτελεσματικός βαθύς ευτηκτικός διαλύτης ήταν το μίγμα γαλακτικού οξέος- δισόξινου κιτρικού νατρίου σε μοριακή αναλογία 15-1 (LA-SCDB15). Η βελτιστοποίηση της διαδικασίας εκχύλισης με πειραματικό σχεδιασμό κατά Box-Behnken και η κινητική μελέτη που εφαρμόστηκε αποκάλυψαν ότι ο βαθύς ευτηκτικός διαλύτης επέδειξε υψηλότερη εκχυλιστική ικανότητα ως προς τις βασικές πολυφαινόλες του φασκόμηλου και ήταν πιο αποτελεσματικός ενεργειακά, σε σχέση με τις προηγούμενες μεθόδους που μελετήθηκαν.

Στο τέταρτο και τελευταίο μέρος, αξιολογήθηκε η μακροπρόθεσμη και η επιταχυνόμενη σταθερότητα των εκχυλισμάτων LA-SCDB15. Σαν δείκτες σταθερότητας χρησιμοποιήθηκαν η δράση κατά ελευθέρων ριζών (A_{AR}) και αναγωγική δράση σιδήρου (P_R). Τα αποτελέσματα και των δύο δοκιμασιών έδειξαν ότι τα εκχυλίσματα ήταν σταθερά υπό τις συνθήκες που δοκιμάστηκαν. Η LC-DAD-MS ανάλυση έδειξε ότι οι βασικές ενώσεις του γένους Salvia παραμένουν αναλλοίωτες από τις συνθήκες της δοκιμασίας σταθερότητας που χρησιμοποιήθηκαν.

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Nomenclature

A_0, Initial absorbance at 515 nm

A_{AR}, antiradical activity (μmol DPPH g^{-1})

A_f, final absorbance

A_t, Absorbance at 515 nm at any time *t*

C_{CD}, cyclodextrin concentration (%, w/v)

C_{DES}, DES concentration (%, w/v)

C_{DPPH}, DPPH concentration (μmoL L-1, μM)

C_{GL} glucerol water proportion

C_{TFn}, Total flavonoid concentration (mg RtE L-1)

D diffusion coefficient

dm, dry mass (g)

***E*,** electric field strength

E_a, activation energy (kJ mol^{-1})

h, initial reaction rate

***k*,** second-order extraction rate constant (g mg^{-1} min^{-1})

k_0, pre-exponential factor (g mg^{-1} min^{-1})

k_1, Second-order rate constant of the first abstracted H-atom (M^{-1} s^{-1})

k_{ref}, second-order extraction rate constant at reference *T* (g mg^{-1} min^{-1})

$P_{R\,(0)}$, Initial reducing power (μmoL AAE g-1 dw)

P_R, reducing power (μmol AAE g^{-1})

R, universal gas constant (8.314 K^{-1} mol^{-1})

$R_{L/S}$, liquid-to-solid ratio (mL g^{-1})

S(s) steering speed (rpm)

T, Temperature (ºC or K)

t, Time (min or days)

$\boldsymbol{T_{ref}}$, reference *T* (°C)

$\boldsymbol{Y_{TFn}}$, Yield in total flavonoids (mg RtE g-1)

$\mathbf{Y_{TP}}$, yield in total polyphenols (mg GAE g^{-1})

Abbreviations

AA, Ascorbic acid

AAE, ascorbic acid equivalents

CD$_{(S)}$, cyclodextrin(s)

DES(s), deep eutectic solvent(s)

DPPH, 2,2-Diphenyl-1-picrylhydrazyl radical

FMAE focused microwave assisted extraction

GAE, Gallic acid equivalents

HBA, Hydrogen bond acceptor

HBD, Hydrogen bond donor

HP-β-CD, hydroxypropyl β-cyclodextrin

LA-SCDB15, lactic acid-sodium citrate dibasic at molar ratio 15:1

LC-DAD-MS, Liquid chromatography-diode array-mass spectrometry

LTTM, Low-transition temperature mixture

MAE, microwave assisted extraction

MAP(s), medicinal aromatic plant(s)

m-β-CD, methyl β-cyclodextrin

NADES(s), natural deep eutectic solvent(s)

PLE, Pressurized liquid extraction

PEF pulsed electric field

PMAE pressurized microwave assisted extraction

RtE, rutin equivalents

SF, Salvia fruticosa

SFE, Supercritical fluid extraction

SLE, Solid-liquid extraction

TPTZ, 2,4,6-Tripyridyl-s-triazine radical

UAE, Ultrasonic assisted extraction

US, ultrasonication

β-CD, β-cyclodextrin

CHAPTER 1. Literature review

1.1 Salvia L. General information

The *Salvia* genus is one of the largest of the *Lamiaceae* family, representing 900 species out of 5600 in total (Topcu et al., 2013; Heinrich et al.,2012)**.** The name of the genus derives from the Latin word "salvare" which means "to save", referring to the healing properties of the plant. Moreover, the name "sage" that is commonly used for *Salvia* species derives from the corrupted *sauge* (sage) in French and *sawge* in Old English and translates to "wise" (Sharma et al., 2019; Ulubelen, 2000)

Most of the species in the Lamiaceae family are herbs or small shrubs with distinct four-angled stems. They have simple opposite or pinnate leaves covered with glandular trichomes. The flowers are zygomorphic with short-stalked epidermal glands. Species of this family are hermaphrodite, with five fused sepals and five generally zygomorphic petals. They consist of four or two stamens, and two very characteristic fused gynaecia, each divided into two partial units developing into a nut with a secondary division into nutlets (Heinrich et al., 2012)

The *Salvia* genus is encountered globally, however, two main centers of origin might be considered, SW and C Asia, and Central America. The largest population of *Salvia* species is located in America (500), almost half of them (275) is located in Mexico while 50 in the USA **(**Σφάλμα! Το αρχείο προέλευσης της αναφοράς δεν βρέθηκε.**)**. In the Old World, 80 species in China, 30 Lebanon/Syria, 22 Palestine, 23 Iraq, 61 Iraq, 23 Afghanistan, 12 Pakistan and lastly, Turkey has the largest population with 87 (Hedge, 1986)**.**

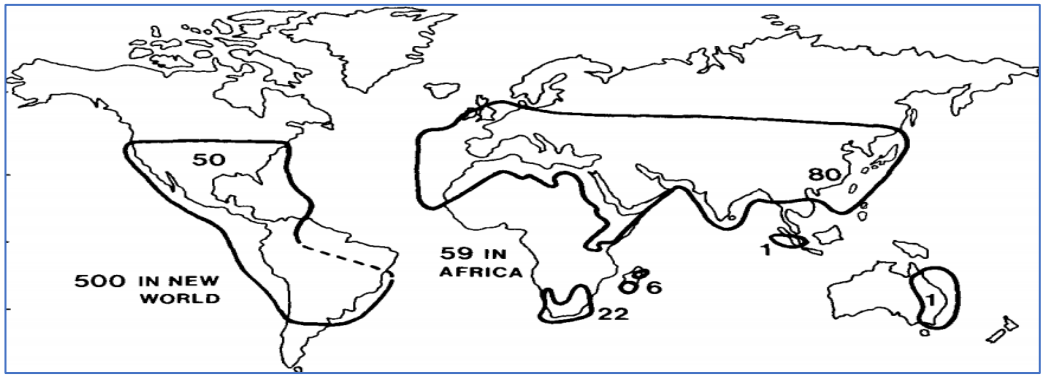

Figure 1.1: Global distribution of Salvia (Hedge, 1986)

In Greece there are 23 species, five of them are grown only in Greece or in Greece and Turkey. Three are mainly located in Balkans and extend in Asia Minor or Italy. Two taxa are distributed in the whole Mediterranean basin and thirteen commonly occur in the Old World and spread in America and Australia (Karousou et al, 2000).

Although the highest population of salvia species occurs in Mexico (250), the center of origin of sage is considered Afghanistan and Central Asia, where lower number of taxa present more diverse morphological characteristics (Paton, 1991). There is no clear evidence on how Salvia was introduced in the Mediterranean coastline, some authors believe that the Greeks at 600 BC spread it through their colonies (Rivera at al., 1994), while others believe it was the Romans who took it from the Egyptians and spread it throughout the empire (Dweck, 2000). There is a plethora of historical data concerning the Salvia genus under the name "Sage" but the specific identity of the plant it's not always apparent (Rivera et al., 1994).

Starting from the classical era, Sage has a long history of use as a medicinal plant. The Romans were using infusions as a stimulant and the Egyptians utilize it to treat infertility. Greeks considered it as a panacea, Hippocrates used to prepare poultices with sage to treat wounds and ulcers (Sharma et al., 2019). Theophrastus mentioned two sage plants, one wild called "sfakon" and another called "elelisfakon". According to Pliny the Elder, the latter was cultivated more often and it was called Salvia by the Romans (Dweck, 2000). In the Middle Ages, healing and cultivation of herbs were the responsibility of monks, and sage was one of the herbs that commonly cultivated in the monastery gardens (Ghorbani & Esmaeilizadeh, 2017). The importance of Sage as a medicinal plant is denoted in many proverbs from the medieval era, an Anglo-Saxon manuscript reads "why should man die when he has sage?", a similar saying exists in Salerno Medical school – "why should man die when he has sage in his garden?". Sage was one of the constituents of the Four Thieves Vinegar. According to the tale, at the time of the plague in Europe, around 1630 AD, four robbers were caught by the authorities stealing from houses that the owners had previously been infected by the disease. Although they were sentenced to death, they manage to save themselves by revealing the recipe of the remedy that they were using for protection before entering the contaminated houses (Dweck, 2000).

Traditionally, sage infusions have been used as tonics to improve cognition and treat diseases of the nervous system. Sage tea has been proved helpful for treating sore throat, colds and also kidney, lung and stomach conditions. It improves digestion and has anti carminative effect. Salvia infusions have been used as an emmenagogue and as an analgesic for joint pains. Dry Sage leaves were ingredients for tooth-powders, as they have been proved efficient for teeth cleaning and gum strengthening (Grieve, 1971)**.** Aztecs used the seeds of the Mexican Salvia, called Chia, to prepare a dye that was used for body paint and ceramic painting. Medically, Chia was used for skin conditions, as a pain reliever and to treat intestinal conditions and fevers (Dweck, 2000).

Besides the medicinal uses, sage also has been used as a spice and a preservative for sausages, ground meats and fish, as a flavoring agent for soups, honey, salads, stews, liquors/bitters, as an ingredient in perfumery and cosmetics, and as a natural insect repellent (Gali-Muhtasib, 2006).

1.1.1 *Salvia Fruticosa* Mill

S.Fruticosa (*SF*) (**Figure 1.2**) is a shrub native to Eastern and Western Mediterranean including Turkey, Middle East, North Africa and Canary Island. According to Kew checklist the species has 19 synonyms with the most common being *S.Libanotica, S.Cypria, S.Lobryana,* and *S.Triloba.* The latter describes the main characteristic of the plant, which is the trilobed leaves (Topcu et al.,2013)**.** The Greek Sage -as *S.Fruticosa* is known in the international trade- is a variable species in terms of morphological characteristics and chemical composition, this fact justifies the plethora of synonyms that the species is known with. Karousou & Kokkini, (1997) observed this variation when they studied samples from different locations from the island of Crete, they attributed this variation to the different climatic conditions and to the topographic diversity of the island**.**

According to Rivera et al (1994), *SF* is known since the fourteenth century BC as it is depicted in the "blue bird fresco" in the house of frescoes, in Knossos. Three-lobed branches were illustrated in Iberian pottery belonging to the 400 BC, a period in which the area was under the influence of Greek civilization. Rivera et al., (1994), considers that the identity of the plant was concealed by ancient authors under the name of "elelisphacon" which was usually referred to as the garden sage (*S.officinalis*), leading to repeated mistakes regarding the information on the species. This claim is supported

by the fact that wild *S.officinalis* rarely occurs south of Balkans and *SF* is the main species in Greece**.**

Figure 1.2: *Salvia fruticosa* (Karousou et al., 2000)

This diversity within the *SF* species was the study subject of (Reales, 2004), in order to address the problem he contacted a multivariate analysis of morphological characters of 99 specimens from the Mediterranean basin. According to his findings, the stems have glandular hairs. Leaves are simple, with one or two small ears, trilobed or even with two pairs of lateral leaflets. Inflorescence can be simple or branched; with glandular hairs and sometimes also stalked glands. The floral bracts excepting the lower verticillaster. The calyx is actinomorphic, tubular, 5–12 mm long; with abundant long (0.6–2 mm) subpatent glandular hairs, abundant glandular hairs, and abundant stalked glands; sessile glands absent; teeth ovate to shortly triangular, 1–3 mm long**.**

Traditionally, SF leaves are boiled and the tea is used for the relief of headaches, stomachaches and abdominal pain. Palestinians use it to treat indigestion and heart disorders. In Turkey it is used against kidney and gall bladder stones, and for the treatment of colds, coughs and influenza. In Lebanon, they prepare a form of cataplasm and apply it externally to heal fractured bones (Gali-muhtasib & Hilan, 2000)**.** In the eastern Mediterranean folk medicine, it is used for the treatment of skin, blood infections, as well as for digestive, circulatory, and respiratory conditions (Giweli et al., 2013)**.**

1.1.2 Bioactive compounds of *S. fruticosa*

The medicinal/bioactive properties that many plants possess are related to a certain group of organic compounds called secondary metabolites. These substances are not essential for the growth and development of the organism but they present a number of functions mostly of ecological nature. As such, some of them are related to the defense mechanism of the plant against predators, other act as attractants of pollinators and some are of allelopathic nature. In contrast, primary metabolites include compounds like lipids, amino acids, phytosterols, etc, that are of paramount importance for the sustainability of the plant (Croteau, Kutchan, & Lewis, 2000)**.** From the qualitative perspective, the chemical composition of the Salvia species is similar for all the members of the section. Variability is observed in the quantities of phytochemicals that authors report, between and within the salvia genus. This can be attributed to the different growth conditions (climate, geographical origin, etc) and extraction procedures (solvents, techniques, etc.).

A total of 145 compounds have been detected up to date in the volatile fraction of SF (Pitarokili et al., 2003). The dominant component of SF essential oil is 1,8 cineole, except for very few cases (Koliopoulos et al., 2010; Pierozan et al., 2009; Longaray Delamare et al, 2007). Other major compounds (>5%) that have been reported often by authors include camphor, α-thujone, β-thujone, α-pinene, β-pinene, β-caryophyllene, and myrcene. The most common method of extraction is hydrodistillation, and the yield reported ranges between 0,25%-5.3% v/w.(Adam et al., 1998; Farhat & Affara, 2001; Giweli et al., 2013; Leontaritou et al., 2020; Pitarokili et al., 2003; Putievsky, 1986; Skoula et al., 2000; Topcu et al., 2013)**.**

The polar constituents of the salvia genus consist of hydroxybenzoic acids, hydrocinnamic acids, flavonoids in their free form or glucosilated, anthocyanins and proantocyanidins. The dominant phenolic compound of the Salvia genus is the ester of caffeic acid with 3,4-dihydroxyphenyllactic acid, called rosmarinic acid (Y.Lu et al., 2002). The polar fraction of SF is understudied and as such, the quantitation reports related to the species are scarce. In the following paragraphs, compounds that have been mentioned in literature at least twice, will be reviewed.

The quantities of caffeic acid that authors report for the *SF* presents the same variability that characterizes the genus. Exarchou et al., (2002) reported trace amounts of caffeic

acid in the acetone and ethanol extracts of SF. Contrarily, Askun et al.,(2009) found that caffeic acid was the predominant compound (7538,6μg/ml) in the methanol extract. Mathe et al., (2010) reported that caffeic acid had the lowest concentration (0,02-0,04 dry wt%) among the phenolic acids in all the salvia species tested in the study. Similarly, Vergine et al., (2019) reported amounts of caffeic acid below the LOQ, in SF and again was the lowest among other salvia species studied.

In SF, ferulic acid is not encountered often. In a seasonal variation study of Papageorgiou et al., (2008), the values reported were between 0.4mg/g – 5,65mg/g, and they were the highest among the hydrocinnamic acids tested. Sarrou et al., (2016), in another seasonal variation study, reported values of ferulic acid in methanolic extracts between 0.002mg/g-0.047mg/g, in this case, the quantities mentioned were the lowest among the other hydrocinnamates.

Coumaric acid is not mentioned often in SF studies, nevertheless, there are some exceptions. Dincer et al., (2012) reported considerable quantities that ranged between 1.349-2.375mg/g dw. Papageorgiou et al., (2008) in a two-year study, quantified coumaric acid in the range of 0.25-0.60mg/g.

Dincer et al., (2012), in a two-year study where wild and cultivated *SF* specimens were analyzed, found minor quantities of chlorogenic acid (0.042-0.149mg/g dw). Sarrou et al., (2016) in a seasonal variation study of cultivated SF found quantities of chlorogenic acid that ranged between 0.009-1.818mg/g dw but also quantified neochlorogenic and cryptochlorogenic acid, 0.001-0.017mg/g dw and 0.001-0.057mg/g dw respectively.

For *S.Fruticosa*, the HBAs reported in literature, are present in their free form. Papageorgiou et al., (2008), in a two-year study, found 5.75mg/g dw of protocatechuic acid in the samples collected in August of the first year, this quantity was the highest among the phenolics quantified. In the same study, the highest amount of vanillic acid, 2.7mg/g dw, reported for the samples collected in February of the first year. Dincer et al., (2012) in a two-year comparison study of wild and cultivated SF, reported quantities that ranged 0.024-0.045mg/g dw for vanillic acid and 0.021-0.033mg/g dw for gallic acid. Sarrou et al., (2016) in a seasonal study found protocatechuic acid (0.030-0.051mg/g dw), p-hydroxybenzoic acid (0.011-0.024mg/g dw), 2,6- dihydrobenzoic acid (0.001-0.009mg/g dw).

Quercetin and its glucosides are present in SF in moderate quantities. Sarrou et al., (2016) reported values for quercetin that ranged between (0.004mg/g-0.101mg/g), for quercetin-3-rhamnoside values between (0.001mg/g-0.003mg/g), for dihydroquercetin between (0.001mg/g-0.016mg/g) and for quercetin-3,4-diglucoside between (0.004mg/g-0.028mg/g). Dincer et al., (2012) found quantities for quercetin between (0.738mg/g-0.755mg/g) and for rutin (0.740mg/g-0.763mg/g).

Sarrou et al., (2016) in a seasonal study, reported minor quantities of kaempferol and dihydrokaempferol, (0.003mg/g-0.054mg/g) and (0.001mg/g-0.011mg/g) respectively, moderate quantities of kaempferol-3-O-glucoside that ranged between 0.014mg/g-0.223mg/g. Dincer et al., (2012) in a comparison study between cultivated and wild plants, reported quantities between 0.600mg/g-0.642mg/g, with no significant differences among the specimens.

Luteolin is a flavonoid that is mentioned quite often in *S.Fruticosa* assays . In a two-year study, Papageorgiou et al., (2008), reported quantities of luteolin that ranged between 0.90mg/g dw and 6.55mg/g dw, with the highest being from the plants that harvested on May of each year. Dincer et al., (2012)**,** found luteolin amounts between 0.898mg/g dw- 1.153mg/g dw, the results showed significant differences between wild and cultivated plants and between the two harvesting years. The storage period (6 months) didn't seem to affect the yield of luteolin. Vergine et al., (2019) reported relatively low quantities of luteolin (0.06mg/g dw) in comparison to other literature existing data, similar amounts detected in other Salvia species in that particular assay. Sarrou et al., (2016), in a 7-month study of two populations, reported quantities of luteolin that ranged from 0.104mg/g - 0.214mg/g dw and also luteolin-7-O-glucoside that ranged between 0.036mg/g – 0.249 mg/g.

1.1.3 Bioactivity of *S.Fruticosa* extracts

The biological activity of salvia species is well studied and many papers reviewed their pharmacological potencies (Ren et al., 2019; Hamidpour et al., 2013; Janicsák et al., 2011; Wang, 2010; Kamatou et al., 2008). Pizzale et al., (2002),compared the antioxidant activity of two origanum species (*O.indercedence, O.onites*) and two salvia species *(S.officinalis, S.Fruticosa*). The crocin-bleaching test showed that *S. fruticosa* had higher antioxidant activity compared to S. *officinalis* but the rancimat test showed no significant difference between the two species. Overall, salvia extracts were stronger

antioxidants than those of origanum. Boukhary et al., (2016), examined the antioxidant activity of the aerial parts and roots of the SF plant, using different solvents such as chloroform, methanol, ethyl acetate, and butanol. The results of the DPPH assay showed that the ethyl acetate extract of the roots had a higher antioxidant activity.

Boukhary et al., (2016)**,** evaluated the anti-inflammatory activity of methanolic extracts of SF against induced paw edema on albino mice. The results showed significantly less paw edema for the two extracts compared to the control while they had almost the same activity compared to the drug. In a similar study**,** Çadirci et al., (2012), found that the n-butanol extract of SF aerial parts exhibited an anti-inflammatory effect.

Perfumi et al, (1991)**,** investigated the hypoglycemic effect of SF infusion on diabetic and healthy rabbits. The results were rather peculiar since single administration didn't lower the blood glucose levels in either healthy or diabetic rabbits but when the treatment repeated for 7 days the reduction of glucose levels was observed only to the diabetic rabbits. The hypoglycemic effect was more intense when the infusion was administered at the same time with an oral glucose load. The authors stated that this is because the SF extract doesn't influence directly the metabolism of glucose but influences its intestinal absorption.

Water extracts of SF have shown to inhibit the proliferation and induce the apoptosis of colorectal cell lines, adding the plant to the list of potential anti-cancer agents (Xavier et al., 2009)**.** Likewise, SF ethanol extracts have exhibited potent antiangiogenic activity on human umbilical endothelial cells (HUVEC) (Zihlif et al., 2013)**.** Methanolic extracts of SF have induced anti-angiogenesis and apoptosis in prostate cancer cells (Atmaca & Bozkurt, 2016)**.**

Elbetieha et al., (1998), investigated the reproductive toxicity of SF extracts (water, ethanol) on male and female rats. The results demonstrated that the ingestion of extracts from female and male rats didn't affect the occurrence of pregnancy. However, the number of implantation sites and the number of viable fetuses were reduced.

In a comparison study between 55 Salvia taxa from Turkey, SF extracts shown the highest acetylcholinesterase activity designating the protective effect of the species against neurodegenerative diseases like Alzheimer's (Şenol et al., 2010). The dose-dependent antiamnesic effect of SF extracts on male albino mice indicates that SF may act as a memory booster (Orhan & Aslan, 2009)**.** Todorov et al., (1984) investigated the

sedative properties of SF, compounds isolated from SF prolonged the hexobarbital sleep in rats**.**

1.2 Extraction of plant metabolites

Plants, besides being a food source, they have been used as flavoring agents, insect deterrents, ornamentals, fumigants, spices, and cosmetics. Folk medicine includes a plethora of remedies, potions, and oils that serve as a palette for the synthesis of new drugs. The first step for the study/utilization of bioactive compounds is their effective removal from the plant matrix (Azmir et al., 2013)**.** The extraction of plant metabolites is dated back to the Mesopotamian and Egyptian era. 250 km south of Baghdad, archeologists discovered clay pots dated from 3500 BC, it is believed that they were used for a kind of extraction similar to a Soxhlet apparatus (Bart, 2011)**.**

In simple words, extraction involves the plant material and usually a liquid (solvent), where the application of energy facilitates the transfer of the compounds of interest into the liquid, in this way it is easier to handle the bioactive components. An efficient extraction procedure provides; removal of the targeted compound from the complex matrix; convert the compounds into a form that they can easily be detected and/or separated; increase the selectivity of the analytical method, increase the sensitivity of the bioassay by increasing the amount of the compound removed; and provide a reproducible procedure that is independent of the sample matrix (R. M. Smith, 2003)**.**

1.2.1 Mechanism of solvent extraction

The mechanism of extraction is governed by two phenomena, equilibrium and mass transfer. Equilibrium concerns the quantity of the solute in the matrix and in the solvent and it is described by the formula:

$$K = \frac{C_s}{C_p} \qquad (1\text{-}1)$$

K=equilibrium constant

C_s= concentration of solute in the solvent

Cp= concentration of solute in the plant matrix

The above relationship shows that the higher the equilibrium constant is, the more of the compound can be dissolved in the given solvent (Gertenbach, 2002). The mass transfer proceeds with the following steps; the plant comes in contact with the solvent,

the solvent diffuses into the particle pores and the compounds are solubilized, the solvated compounds are diffused out of the pores and enrich the bulk solvent with the solute (**Figure 1.3**).

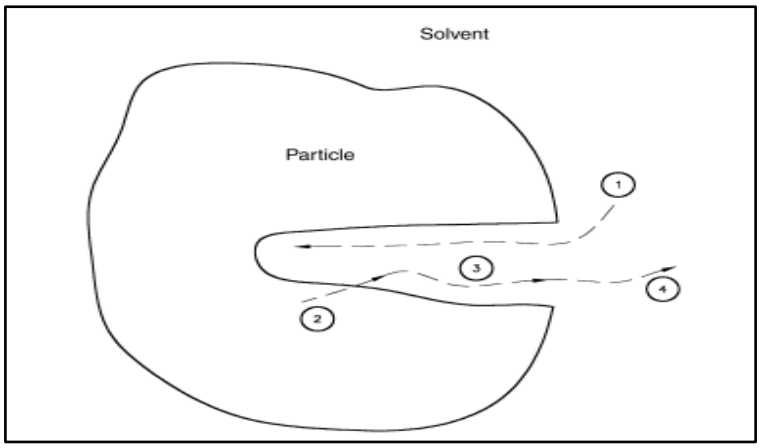

Figure 1.3: Four steps for mass transfer: (1) solvent soaks into the pores within the particle, (2) solute dissolves into the solvent within the pores of the particle, (3) dissolved solute migrates to the surface of the particle, and (4) dissolved solute at the particle surface diffuses into the bulk solvent (Gertenbach et al., 2002).

The diffusion step is the rate-determining step and it is governed by Fick's Second Law, described by the following formula:

$$\frac{\delta C}{\delta t} = D\frac{\delta^2 C}{\delta x^2} \qquad (1\text{-}2)$$

The *C* represents the concentration of the solute and *t* the time, *D* is the diffusion coefficient and *x* is the particle diameter. Briefly, the formula demonstrates the relation of the extraction rate with the particle size, the concentration gradient, and the diffusion coefficient. It has been observed that the extraction rate is rapid at the beginning where the concentration gradient is high and diminishes as the equilibrium is approached. By renewing the solvent equilibrium is delayed and the diffusion proceeds faster, so the extraction moves swiftly (Harouna-Oumarou et al., 2007, Gertenbach, 2002). Smaller particle size increases the surface area which the solvent is in contact with, thus decreases the pore diffusion path. In the case of plant material where leaves are extracted size has the meaning of thickness. The solvents composition is also important, pH, polarity, etc. are evenly affecting the extraction rate, "like dissolves like" is the proverb that applies here. The liquid to solid ratio correlates with the concentration of the solvent in the outer surface of the particle. Higher values imply a higher concentration gradient

between the solute's concentration at the surface of the particle and inside it. High concentration gradient leads to rapid extraction of phytochemicals. Temperature affects both, the yield of the extraction and the mass transfer rate. High temperatures increase the solubility of compounds and therefore result in a higher equilibrium constant. At the same time, increasing temperature results in higher diffusion rates, thus a higher extraction rate. Consideration should be made when the targeted compounds are thermolabile. (Wongkittipong et al., 2004; Gertenbach, 2002).

1.2.2 Sample preparation

Before an extraction is employed, preparation of the plant samples is recommended to preserve the phytochemicals and facilitate the procedure.

1.2.2.1 Drying

After harvesting, the plant material is prone to enzymatic reactions and fungus attacks, which are facilitated by moisture. As such, drying is an important step unless the plant material is going to be processed soon after collection. Air drying is the simplest and cheapest method of dehydration. Depending on the plant tissue and the ambient temperature, it can take from 3-7 days to a month. High temperatures may be employed to expedite the procedure but caution should be made concerning thermolabile compounds. For this purpose, conventional or microwave ovens might be used, in the first case thermal energy removes moisture, and in the second electromagnetic radiation is employed to dry the sample. Freeze-drying is a method based on sublimation. The sample is initially frozen to solid, then it is placed in a chamber under vacuum where lyophilization occurs. With this method, phytochemicals are preserved but it is also complicated and expensive, hence it is recommended for delicate and high-value materials (Azwanida, 2015).

1.2.2.2 Milling

An important step before the extraction of phytochemicals is the reduction of the plant tissue size. Small particle size increases the surface contact between the solvent and the substrate, at the same time, it reduces the diffusion distance of the solute within the solid, increasing the mass transfer rate from the solid to the liquid solvent. The size reduction is feasible through the use of simple mortar and pestle or more advanced milling apparatuses and blenders (Azwanida, 2015; Z. Wang, 2011). Many studies where optimization of the extraction parameters was investigated concluded that among

the significant factors affecting the procedure is the particle size (Tchabo et al., 2018; Jovanović et al., 2017; Ćujić et al., 2016). However, very small particle size may lead to strong cohesive forces between the particles and the subsequent agglomeration of them, in this case, the contact surface area is decreased leading to lower extraction yield (Nejad-Sadeghi et al., 2015). Moreover, Hu et al., (2012), observed that superfine powder of tea leaves gave lower total phenolic and catechin content, he attributed the fact to the elevated temperatures produced from the milling apparatus.

1.2.3 Conventional solid-liquid extraction (SLE) techniques

1.2.3.1 Maceration

The simplest and oldest extraction method is maceration. In this procedure, the powdered plant is immersed in the extraction solvent and remains there for at least 3 days, where it is agitated regularly. Then the solvent is decanted and the plant material is strained, then it is pressed to remove the extra solvent, eventually, the two liquids are combined to a single fraction which finally is clarified. The process is slow with low efficiency and requires large quantities of solvent. Nevertheless, it is suitable for the extraction of thermolabile components (Zhang et al., 2018; Handa, 2008).

1.2.3.2 Infusion

In this case, the plant material remains in the solvent for a short period, thus the resulting solution is more dilute in comparison to the maceration product, hot or cold solvent may be used (Handa, 2008).

1.2.3.3 Digestion

Similar to maceration but gentle heat is applied. This method is used when improved efficiency of the solvent is needed and heat is not an obstacle (Handa, 2008).

1.2.3.4 Decoction

This method involves the boiling of the plant material- usually with water-into a specific volume of solvent (1/4, 1/16 ratio) for a certain amount of time. The procedure is complete when the remaining volume of solvent is ¼ its initial, concentrated solutions result from this method. The method is not suitable for thermolabile or volatile compounds (Zhang et al., 2018; Handa, 2008).

1.2.3.5 Percolation

For this method, a specific container is used (percolator), it is cone-shaped and opened at top and bottom. The procedure involves the plant material soaking in a defined amount of solvent for approximately 4h. After that, the plant material is packed and more solvent is added at the top of the plant mass. The outlet of the percolator is opened and the extract drips slowly, a sufficient amount of solvent is added periodically as seen fit. Percolation is more effective than maceration because it's a continuous process where the saturated solvent is replaced by fresh solvent (Handa, 2008; Q.-W. Zhang et al., 2018).

1.2.3.6 Cold fat extraction (Enfleurage)

This is a traditional extraction method of volatiles that is still used nowadays and it was popular in traditional perfumery. The principle of the method involves the extraction of fragrances mainly from flower petals, with the use of fat. The flower petals are placed on the top of the fat which already has been applied in large trays. The volatiles are absorbed from the fat and a new batch of fresh flowers replace previous until the fat is saturated with flower oil. Finally, the oil is extracted with alcohol and isolated. The success of this method is heavily dependent on the quality of the fat employed. It needs to be of high purity, odorless and of defined consistency. The procedure proceeds in cool cellars temperatures and the fat must be prepared according to them ensuring the appropriate texture of the tallow (Handa, 2008).

1.2.3.7 Heat reflux extraction

In this method, the plant material is immersed into the solvent of choice, and heat is applied to the extraction flask. A condenser is attached to the neck of the flask facilitating the reflux of the solvent back to the container. High temperatures and volatile solvents are allowed since the condenser doesn't permit the depletion of the extractant. The method is not suitable for the extraction of thermolabile molecules. The procedure is rapid and more efficient than percolation and maceration since the driving force of the extraction is temperature, which increases the diffusion and solubility of the compound of interest (Q.-W. Zhang et al., 2018).

1.2.3.8 Soxhlet extraction

Soxhlet extraction is one of the oldest and most popular techniques for the recovery of plant metabolites. It was developed in 1879 by the German chemist Franz Ritter Von

Soxhlet for the determination of fat in milk, since then, it has been used as a model for the comparison of new extraction methods (Jensen, 2007).

The powdered sample is placed in a thimble made from permeable material on the top of a flask filled with the extraction solvent. The solvent is heated by a heating plate/mantle and it distills into the thimble. The sample is macerating in the thimble and when the amount of solution reaches an overflow level it is aspired by a siphon back to the balk liquid. The solutes remain into the flask and the distilled solvent returns to the thimble with the plant material. The procedure runs repeatedly until the extraction is completed (Azmir et al., 2013). The method is considered exhaustive, the plant material comes in contact with fresh solvent repeatedly, so the system is brought constantly to high transfer equilibrium. The procedure is simple and it doesn't require any advanced training. The equipment is not sophisticated and of low cost. It allows the extraction of higher quantities of plant material than most of the recent methods with good recovery and without the need for filtration. The method is limited to the extraction of low and medium volatility compounds while the long-time presence of the solutes in high temperatures restricts its use to thermostable biomolecules. It consumes large volumes of solvents and it is energy demanding (water cooling, heating, etc). The process is time-consuming and of low efficiency since the solvent comes in contact with the plant material at low temperature after its condensation. Similarly, no agitation occurs, meaning the solvent in the outer surface of the plant matrix is not renewed thus a lower equilibrium ratio is observed (Luque de Castro & Priego-Capote, 2010; Romanik et al.,, 2007). The low efficiency of Soxhlet extraction led manufacturers to utilize emerging technologies to improve methods performance. High-pressure Soxhlet extractor consists of a conventional Soxhlet apparatus inside a high-pressure stainless-steel container, a water bath, and an oven. The extractants used are volatile organic solvents or carbon dioxide. The pressure builds in the extractor by raising the heating temperature and it might reach 1200 psi. The method is faster in comparison to the conventional and provides better recovery for the light-sensitive compounds (Ndiomu & Simpson, 1988). The employment of ultrasound energy to the conventional Soxhlet extraction has been investigated with positive results. The device is similar to the conventional but includes a thermostated bath in which the Soxhlet container remains and sonication is applied by a probe. The extra energy by the ultrasonic waves accelerates the extraction procedure significantly (Luque-García & Luque de Castro, 2004). The most successful attempt to

enhance the performance of the Soxhlet method was achieved by the incorporation of microwave energy into the procedure. The apparatus in its final version consists of a conventional Soxhlet assembly where the thimble compartment is inside a conventional microwave oven. In this set-up, 2 energy sources are utilized, heating for the solvent compartment and microwave energy for the sample compartment. The whole procedure (extraction cycles, siphoning, distillation, etc) is automated allowing full control of the procedure. The advantage of this device is that reduces the extraction time significantly (Luque de Castro & Priego-Capote, 2010).

1.2.3.9 Hydrodistillation

Hydrodistillation is one of the oldest isolation techniques, distillation apparatuses date back thousands of years (Schmidt, 2015). The method is suitable for the extraction of volatile biomolecules (essential oil) and the solvent used is water. Three physicochemical processes govern the hydrodistillation procedure; hydrodiffusion, hydrolysis, and decomposition by heat. Depending on the set-up of the distiller, three types of distillation exist; water distillation, water, and steam distillation, and direct steam distillation.

In the first case, the plant is immersed in an adequate volume of water and it is allowed to come to boil. The water vapors together with the oil droplets pass through a condenser and finally separated in an appropriate container. A disadvantage of this method is that due to the direct contact of water with the plant material, hydrolysis of certain compounds (esters, etc) occurs, meanwhile, oxygenated components cannot be fully recovered since they tend to remain in the water.

In water and steam distillation the plant material is separated from the water, either in different containers or in the same but kept separately by a grid. This method has a higher oil yield and it is rapid, the quality of the oil is better if the conditions of the distillation are controlled carefully.

In direct steam distillation, the boiling water is in a separate container (boiler) and only steam comes in contact with the plant material. The temperature in the plant chamber does not exceed 100 ^{0}C and the steam supply can be readily controlled. The main advantage of this method in that there is no hydrolysis or thermal decomposition of the oil constituents, yielding a product of high quality (Handa, 2008).

1.2.4 Advanced extraction techniques

1.2.4.1 Supercritical fluid extraction (SFE)

The extraction properties of supercritical fluids were known since the end of the 19th century but the first application came from Zosel (1964) who first presented a patent for the decaffeination of coffee using SFE (Azmir et al., 2013). Supercritical is the state where the substance exists beyond its critical point. This point is defined by specific temperature and pressure above which, the liquid and gas states of the substance are not distinct. Supercritical fluids share properties from both the liquid and the gas phase. They have a liquid-like density meaning similar solvation power to liquids, and gas-like viscosity meaning gas-like diffusion properties. These properties can be modified by increasing the temperature and the pressure offering some specificity to the process (Chemat et al., 2020)**.**

The most common extractant used in SFE is CO_2 because of the many benefits that offer; it has a low critical temperature (31^0C) and a low critical pressure (73bar), which is important for the preservation of bioactive molecules and at the same time these values are considered user-friendly as they can be modified easily and offer some selectivity (Azmir et al., 2013)**.** Moreover, it is readily available, it is cheap, it is inert, it has low toxicity and it can be recycled. The only draw-back is its low polarity which makes it ideal for essential oil and lipid extraction but not for polar molecules. To overcome this obstacle, polar compounds can be added in small amounts acting as modifiers to improve the solvent properties**.** A basic SFE set-up includes the following parts; a tank with the main solvent, usually CO_2, a pump that pressurizes the solvent, a second tank with the modifier and its pump, a mixer to combine the two solvents, a thermostated oven (autoclave) where the pressurized solvent enters and the extraction occurs, and a trap where the extractant separates from the solvent (**Figure 1.4**) (Khaw et al., 2017; da Silva et al., 2016).

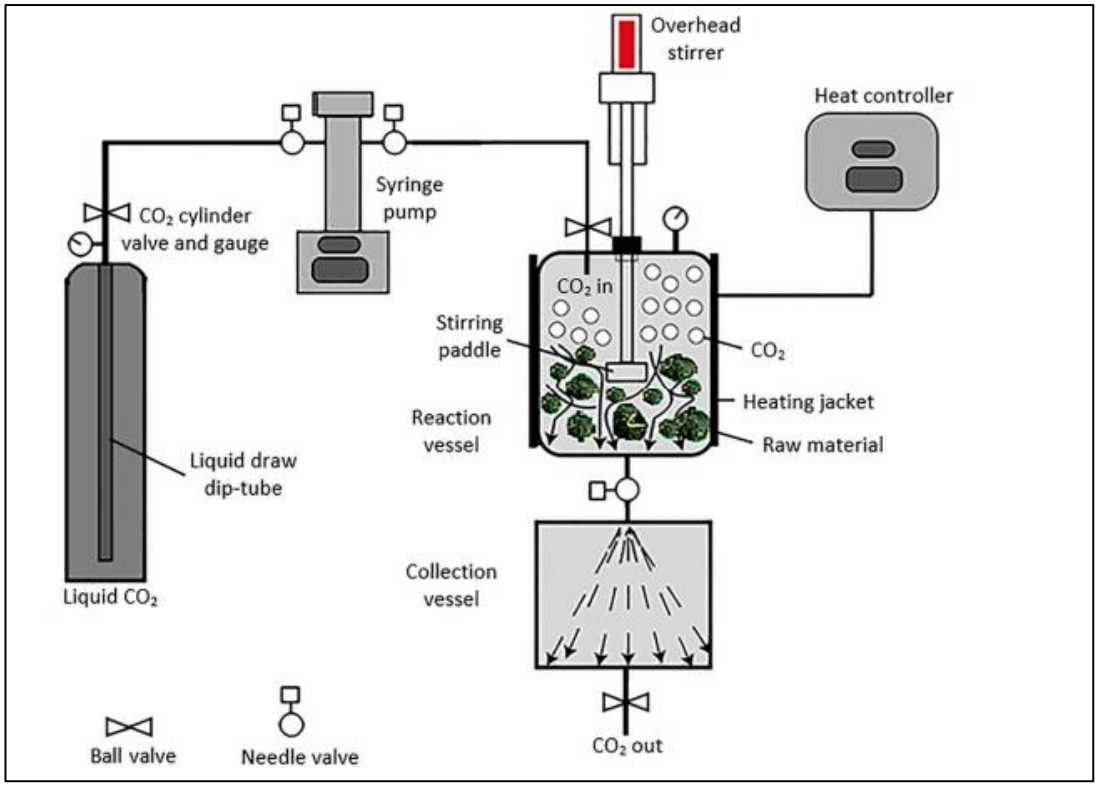

Figure 1.4: A typical supercritical fluid extraction system (Khaw et al., 2017)

The method offers the following advantages when compared to conventional methods. (1) In the supercritical state, the solvent has a higher diffusion coefficient and better solubilizing properties, as such, the extraction proceeds faster than common methods. (2) The solvent can be recycled and reused (reflux), providing a complete, cheap, eco-friendly extraction method. (3) By altering the temperature and the pressure the selectivity of the solvent can be tailored. (4) The separation of the solute from the solvent is easier and faster by just depressurizing the supercritical fluid. (5) The temperatures used are relatively low, thus the thermolabile compounds are preserved better (6) CO_2 and the low quantities of modifiers used are considered safe and environment friendly. (7) The apparatus can be coupled with analytical instruments like gas chromatography detectors (Azmir et al., 2013).

1.2.4.2 Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE) method developed back in 1995 from Dionex initially for the extraction of organo-halogenated pollutants from soil samples (Dean, 2010). In this method, the sample -usually solid- and the solvent, are inside the extraction container where medium temperature (50-200^{0}C) and pressure (500-3000psi)

are applied for a short period (5-10min). The process is similar to the supercritical fluid extraction, but the solvent, in the case of PLE, remains well below the critical values as far as temperature and pressure concerns (Richter et al., 1996). The temperature accelerates the process while the pressure keeps the solvent into the liquid state even when the working temperature is well above its boiling point. At these conditions, the liquid has enhanced solvation ability and lower viscosities thus better diffusion properties**.** As a result, the solvent enters freely into the matrix pores and contact the analyte, improving the extraction rate**.** Although the usual extractants are organic solvents and mixtures of them, water has been also used but at lower temperatures and pressure than the critical ones. Under these conditions (subcritical), water behaves similarly to organic solvents therefore it can extract a wider spectrum of compounds (R. M. Smith, 2003)**.**

Although the method was initially invented for removal of soil contaminants, it has been used successfully for the extraction of phenolic compounds, carotenoids, and essential oils from food matrices (Mustafa & Turner, 2011)**.** Recently, (Liang et al., 2020) reviewing the application of PLE to herbal analysis, he noticed that out of the 189 publications, concerning herbal screening studies in the period 2003-2019, only two of them used PLE as an extraction method. He ascribed the reason to the co-extraction of compounds like lipids and chlorophylls, which interfere with the analytical assays.

Application of high temperature and pressure during the extraction enhances the yield but also accelerates the process while using minimal amounts of solvent. Moreover, the equipment set-up protects sensitive molecules from oxygen and light. Because of the high temperatures involved in the process, consideration should be given when the method is used for the extraction of thermolabile compounds. Additionally, reaching high pressures requires expensive equipment so the investment should be compensated by the profits of its intended application (Mustafa & Turner, 2011)

Another extraction method utilizing high temperature and pressure was proposed by Allaf back in the 1990s and it is named “Instant Controlled Pressure Drop”. Typically, the plant material is subjected to elevated pressure (up to 1MPa) and temperature (up to 180 ^{0}C) for a short time (5-60 sec), followed by an abrupt pressure drop to vacuum (3-5kPa, Δt=20-200ms). Under those conditions, the plant material suffers significant mechanical stress, and events such as water auto-evaporation, instantaneous cooling of

the sample, and swelling occur simultaneously, causing the rupture of the cell membranes and the release of plant metabolites. The aforementioned phenomena and the use of water vapors as solvents qualify the method as a green extraction technique. Meanwhile, the limited time that the plant material is subjected to high pressure and high-temperature conditions, makes this method suitable for the extraction of thermolabile compounds (Chemat et al., 2020). The method has been effectively used as a pretreatment for the extraction of phenolics from grape powder (Sánchez-Valdepeñas et al., 2015) and bio-oil from sunflower and castor seeds (Eikani et al., 2019)**.** The method has been proved more efficient and rapid for the extraction of essential oils compared to the conventional hydrodistillation technique (Kristiawan et al., 2008)**.**

1.2.4.3 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction utilizes the ultrasonic waves for the effective removal of compounds from solid matrices. The ultrasonic waves are sounds with frequencies above the human range, starting from 20kHz up to 100MHz. According to range and application, ultrasound can be divided into three distinct regions; power ultrasound (20kHz-100kHz), extended range (100kHz-2MHz), and power ultrasound (2MHz-10MHz). The first two usually find application in cleaning, welding, and chemistry (sonochemistry) while the latter is used in medical imaging (Ojha et al., 2020). Sound waves when they pass through gas or liquid behave like longitudinal waves and as such, they propagate into the medium through repeated compression and expansion cycles. When the molecules are pulled away (expansion) negative pressure is built, and if the intensity is high enough, bubbles or cavities are formed in the liquid. These bubbles cannot absorb the energy supplied from the ultrasound and eventually collapse. This procedure produces extremely high temperatures (5000^0C) and pressures (2000atm) (**Figure 1.5**). The bubbles collapse in a pure liquid is uniform but when a solid surface is nearby the implosion is asymmetric and jets of liquid are formed reaching speed up to 400km/h. These micro-jets have a detrimental effect on the surfaces of solids (Luque de Castro & Capote, 2007)**.**

Cavitation phenomena explain the extraction potential of ultrasound through multiple mechanisms. The formation of micro-jets and the shockwaves produced from the implosion of cavitation bubbles explains the fragmented particles that a lot of authors observed after the application of ultrasound. These findings were attributed to the

increased penetration capacity of the solvent to pores and canals after ultrasound treatment. During sonication, shear forces are generated which results from the oscillation and collapse of cavitation bubbles within the liquid. The latter effect is rather useful in applications such as mixing or emulsification. The enhanced extraction efficiency of ultrasonic irradiation is not the result of one of the previous effects but the outcome of their combined action (Chemat et al., 2017).

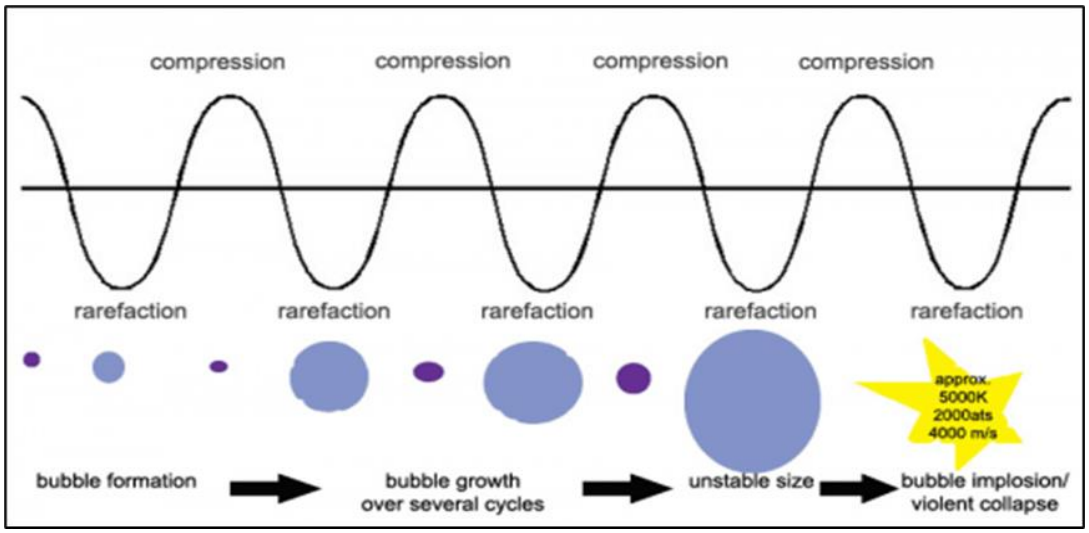

Figure 1.5: Formation of cavitation bubbles (https://www.hielscher.com/wp-content/uploads/ultrasonic-cavitation-bubble-collapse-hielscher.png)

Parameters affecting ultrasonication efficiency include; power, frequency, the shape of container, temperature, solvent, and liquid to solid ratio. It is known that the lower the frequency the larger the cavitation bubble (Esclapez et al.,2011). The usual working frequencies are in between 20kHz-100kHz, in this range, increasing frequency facilitates the extraction of polyphenols (Paini et al., 2016). When higher frequencies used (500kHz) an opposite effect was observed where less plant tissue damage was noticed the plant material (Toma et al., 2001)**.** In general, increasing the power of ultrasonication increases the extraction efficiency of polyphenols, due to enhanced cavitation effect. Considerations should be taken when high frequencies and power are used because of the formation of free radical and their adverse effect on polyphenols. This phenomenon is intense when oxygen is dissolved in the solvent medium or water is present (Dzah et al., 2020)**.** High temperatures induce a decrease in surface tension and increase in vapor pressure, as such, more solvent vapors enter the cavitation bubbles and they collapse less violently reducing the cavitation effect**.** The effect of the solvent

is subjected to the same factors previously mentioned, like vapor pressure, presence of water, etc. Dissolved gasses, though, act as nuclei and facilitates the formation of bubbles, thus increase the cavitation effect**.** High viscosity solvents demand higher energy to initiate the cavitation phenomenon since the intramolecular forces are higher (Chemat et al., 2017). The shape of the container and the type of reactor also affect the efficiency of the sonication process. The use of probe is favored since in the sonication bath the waves should pass through two media (water and solvent), and as a result, the energy affecting the solid is less. In the case of the sonic bath, the volume of water is also important while the shape and the immersion depth of the probe also seem to affect the procedure (Esclapez et al., 2011)**.** The shape of the extraction vessel affects the sonication procedure since the waves are reflected from solid surfaces, flat bottom vessels are preferred (Chemat et al., 2017)**.** The solvent to material ratio also influences the sonication procedure in the sense that concentrated mixtures inhibit the propagation of the sound waves through the mass of the fluid (Dzah et al., 2020)**.**

UAE is a physical extraction method that demands less solvent and less time in comparison to conventional techniques. The procedure is versatile and can be easily adapted to the industrial level. Nevertheless, multiple factors should be controlled carefully to avoid degradation (radical formation, thermal) of the compounds of interest.

1.2.4.4 Microwave-assisted extraction (MAE)

The use of microwave energy in laboratory applications was first introduced back in 1975 for the digestion of organic matter by (Abu-Samra et al., 1975). The first study, though, where microwave irradiation applied for the extraction of organic compounds, came 11 years later from (Ganzler et al., 1986). Microwave irradiation uses an electromagnetic field at high frequencies ranging from 300MHz to 300GHz (H. Wang et al., 2016)**.** The working frequency for chemical applications is around 2.45GHz, in this range, the result of the absorbed microwaves is the production of heat (dielectric heating). The prerequisite for that is the treated substrate contains water or other polar molecules (Kapoore et al., 2018)**.**

Dielectric heating is the result of two mechanisms; dipole rotation and ionic conduction **(Figure 1.6)**. In the first case, the dipoles under the influence of polar components and the effect of the applied electric field, they try to rearrange their position, this molecular movement produces heat. In the latter, the dipoles, flow into the irradiated media under

the effect of the electromagnetic field and the resistance they meet produces friction and consequently heat. As such, the production of heat is related to the polarity of the molecules and their dielectric constant (Veggi et al., 2013).

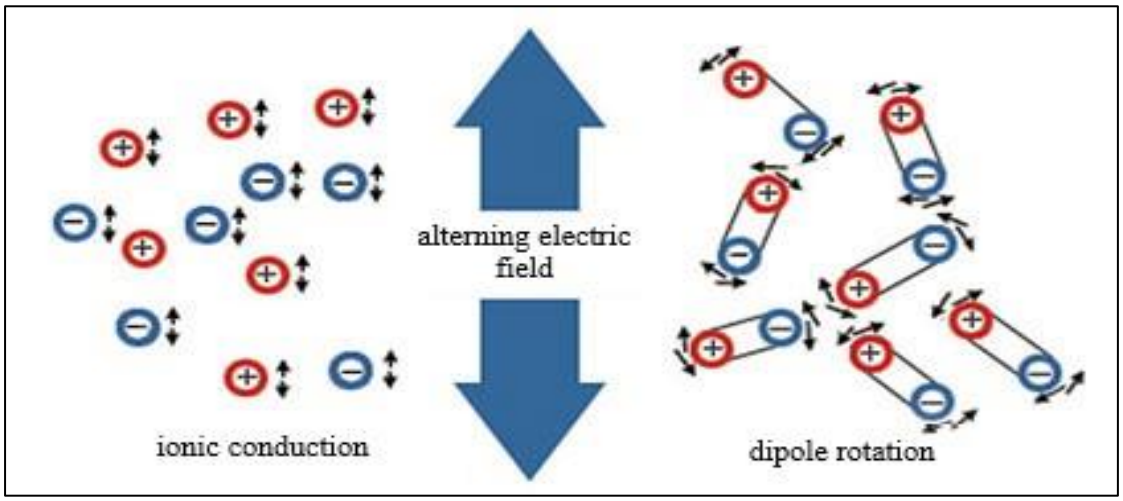

Figure 1.6: Mechanism of dielectric heating (https://www.pueschner.com/images/content/grafiken/alterning-elec-field-en.jpg)

Although the main extraction mechanism in MAE is through the thermal effect similar to conventional solvent heating, there are some differences to consider. In the case of conventional heating, the sample is heated through conduction with the media (solvent). During microwave irradiation, the sample is heated due to the oscillation of polar compounds already present in it. For the extraction of plant metabolites, when moisture absorbs the microwave energy, the water inside the cells start to heat up and eventually evaporates. Evaporation creates high pressure which results in the rapture of the cell wall and the leaching out the solutes to the solution (Mandal et al., 2007). Parameters that affect the efficiency of MAE are the dielectric constant of the solvent and the power of the microwave oven. Water added in a low polarity solvent can improve its extraction capacity by increasing the penetration into the substates pores. The addition of salts (buffers) to the mixture increase the heating rate since they induce polarization. Microwave power and temperature are correlated since the increase in power results in higher temperatures and higher extraction efficiency (Veggi et al., 2013).

Two technologies are employed named pressurized MAE (PMAE) and focused MAE (FMAE). The first consists of a magnetron (microwave source) and an oven where the sealed extraction vessel is on a rotatable disc and temperature and pressure are

controlled. Multiple extraction vessels are possible. In the FMAE system, the magnetron with a focusing device targets the microwaves only to the extraction vessel. The system is a safer alternative to the "close" and high-pressure PMAE, at the same time it can be mounted in Soxhlet type apparatus facilitating the distillation The advantages of the MAE include; short extraction times because of the phenomenon of dielectric heating, it can be coupled with analytical devices, allows the treatment of multiple samples. Nevertheless, it is limited by the use of polar solvents (H. Wang et al., 2016).

1.2.4.5 Pulsed electric field (PEF) extraction

PEF technology involves the application of short electric pulses of high energy to a product placed between two electrodes. The resulting permeability of biological membranes after the application of alternate electric field was known since the beginning of the 20th century, nevertheless, thorough work was initiated during the 1990s. Applications of high-intensity PEF concerned food preservation studies where microbial destruction and enzyme inactivation was needed. Low-intensity PEF concerned applications where soft treatment was desired such as extraction and drying (Soliva-Fortuny et al., 2009). The method is based on the phenomenon of electroporation. Although several explanations have been proposed for the description of electroporation, the theory of aqueous pore formation has been widely accepted. The cell membrane consists mostly of phospholipids forming a bilayer. Because of the nonpolar nature of its interior, it is considered almost impenetrable for polar molecules. Nevertheless, water passes through at rates that cannot be explained by simple diffusion. Under certain conditions of temperature and surface tension, this permeation can be explained by the formation of tiny pores (<1nm) with a lifetime of a nanosecond. According to aqueous pore formation, the application of an electric field to the lipid bilayer reduces the energy needed for the spontaneous formation of these pores and as a result, more pores are formed, more often and they last more (milliseconds to minutes). During the formation of the pores, the polar headgroups of the lipid bilayer are rearranging themselves to the polar water molecules. (Kotnik et al., 2012; Weaver & Chizmadzhev, 1996).

The parameters affecting the process include electric field strength and treatment time. The electric field strength refers to the electric field locally applied between two electrodes and it's calculated by the voltage applied across the electrodes divided by the distance between them. The treatment time depends on the number of pulses delivered

and the specific energy. The latter is expressed in kJ/kg^{-1} and it represents the electrical energy needed for the generation of a high voltage pulse in the treatment chamber. The total specific energy required for a process is calculated by multiplying the total number of pulses by the specific energy per pulse (Martínez et al., 2018).

The advantage of PEF is that it is a non-thermal technique, meaning that the procedure doesn't increase the temperature of the system significantly and thermolabile compounds are not affected. Moreover, the process affects only the cell membrane, thus enhancing the selective extraction of intracellular components and at the same time the membrane permeability is reversible denoting the non-destructive nature of the procedure (Martínez et al., 2018)

1.2.4.6 Enzyme assisted extraction (EAE)

Plant origin biomolecules are dispersed in the cytoplasm or they are retained by the polysaccharide-lignan complex, as such, they are not easily accessible to the extraction solvents. To overcome this obstacle, enzymatic reactions have been used as a pre-treatment, increasing the extraction yield of the conventional solvent extraction. The addition of specific enzymes like amylase, cellulase, and pectinase disrupts the cell wall and hydrolyzes the structural components of the plant tissue, facilitating the penetration of the common solvents (Azmir et al., 2013). For the enzymes to be used effectively, it is important a good understanding of their mode of action, their specificity, and the operating conditions for the selected plant material. The parameters affecting the procedure are these that affect the enzyme functionality in every reaction. In every enzymatic reaction, the substrate must have unhindered access to the specific active site of the enzyme. As such, the main factor for the successful binding of the substrate to the enzyme is the tertiary structure of the enzyme. The tertiary structure of the enzyme is affected by factors like temperature, pH, and ionic strength of the environment. (Sowbhagya & Chitra, 2010). For effective usage of the EAE method, it is important prior knowledge of the cell wall composition and the structure of the solute, for the correct enzyme to be selected. Phenolics usually are entrapped between the polysaccharide chains of the cell wall, such as cellulose, hemicellulose, and pectin. Phenolic acids, form ether bonds with the hydroxyl groups of lignin, and tannins form complexes with proteins. Hence, cellulase, hemicellulose, and proteases are employed to solubilize the plant cell wall and release the intracellular biomolecules. The pH should be in the working range of the enzyme; however, consideration should be made that

many proteins might be insoluble in that range and may hinder the biomolecule release. The temperature should be maintained at the proper levels. High operating temperatures cause lower enzyme activity and at the same time, inactivation of proteins and other biomolecules (Nadar et al., 2018).

The extraction of biomolecules by enzymatic pretreatment offers higher extraction rates, increased yield, and reduces the use of solvents. The enzymatic reactions usually proceed at low temperatures, thus the extraction of thermolabile compounds is feasible. However, the method has some drawbacks; (i) enzymes cannot completely hydrolyze the plant cell wall limiting the yield of the extraction; (ii) The cost of enzymes for large scale extractions is very high; (iii) Scaling up an enzyme assisted extraction is a challenge since new factors like environmental, dissolved oxygen, etc, are taking over and alter the enzyme activity (Puri et al., 2012).

1.2.5 Hybrid techniques.

As the need for novel more efficient extraction techniques was growing, many researchers investigated the possibility of combining different extraction methods and their unique modes aiming at higher yields and shorter extraction times. (Luque-García & Luque de Castro, 2004), developed a method where a thermostated bath with an ultrasound probe was attached to the extraction chamber of a Soxhlet apparatus combining the advantages of Soxhlet extraction- recycling of solvent- with the enhanced mass transfer of ultrasounds **(Figure 1.7)**.

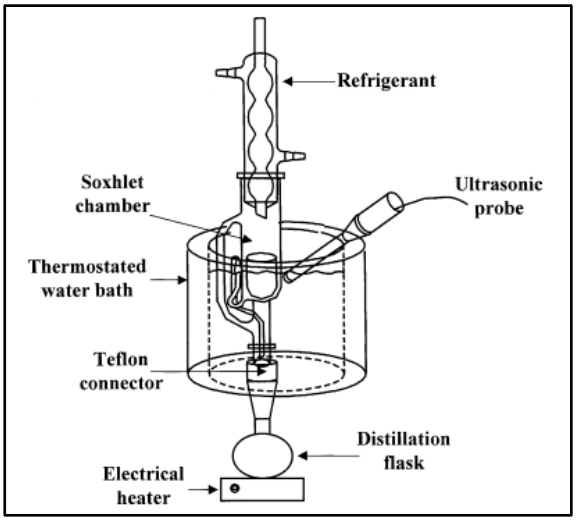

Figure 1.7: Combination of Soxhlet apparatus with ultrasound probe (Luque-García & Luque de Castro, 2004)

Pingret et al., (2014), combined a Clevenger apparatus used for the distillation of essential oils with an ultrasound probe immersed in the boiling flask. The results showed that the oil composition was similar to that from the conventional distillation but the extraction rate was 4 times faster **(Figure 1.8).**

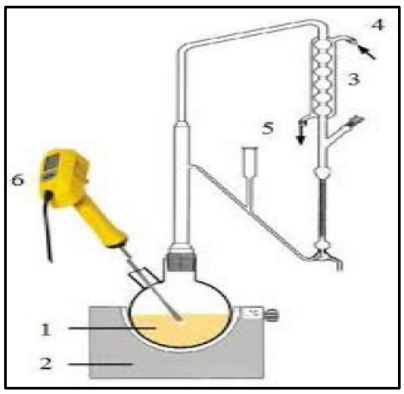

Figure 1.8: Combination of Clevenger apparatus with ultrasound probe (Boubechiche et al., 2017)

Sumere et al., (2018), investigated the simultaneous effect of high pressure, high temperature and ultrasounds on the extraction of phenolics from pomegranate peels. The authors concluded that the process provides enhanced extraction yield of phenolics**.**

In another study, the efficiency of simultaneous microwave irradiation and ultrasound was investigated for the extraction of bioactive compounds from brown microalgae. The results showed that the combination of the two technologies gave higher yields in comparison to the single treatment of each method. In the same study, the yield of MAE was 10-fold higher than that of UEA (Garcia-Vaquero et al., 2020)**.**

Allaf et al., (2013), investigated the effect of DIC pretreatment of orange peels and their sequential extraction by microwaves. The HPLC analysis showed that the kinetics and yields for the recovery of hesperidin and naringenin were the highest when compared to sequential hydrodistillation and conventional solvent extraction**.**

Balachandran et al., (2006) examined the concurrent use of ultrasound and supercritical CO_2 during the extraction of compounds from ginger. During the experiment, an ultrasound transducer was externally attached to the extraction vessel and the system

was inside a thermostated water bath **(Figure 1.9).** Initially, SFE proceeded, and later on ultrasonication applied to the sample in the extraction vessel. The yield was significantly increased by 30% compared to single SFE**.**

The synergistic effect of ultrasonication and enzymolysis for the extraction of polysaccharides from pumpkin has been investigated by Wu (2014), all the combined modes of enzyme and ultrasonication exhibited higher yields compared to the single treatments (Wu et al., 2014)**.** From the previous, someone may notice that ultrasounds are the most popular energy source to use in conjunction with other techniques for the efficient extraction of phytochemicals. This may be attributed to the technical characteristics of an ultrasound transducer, the relative safety of ultrasound waves, and the simple incorporation into other systems.

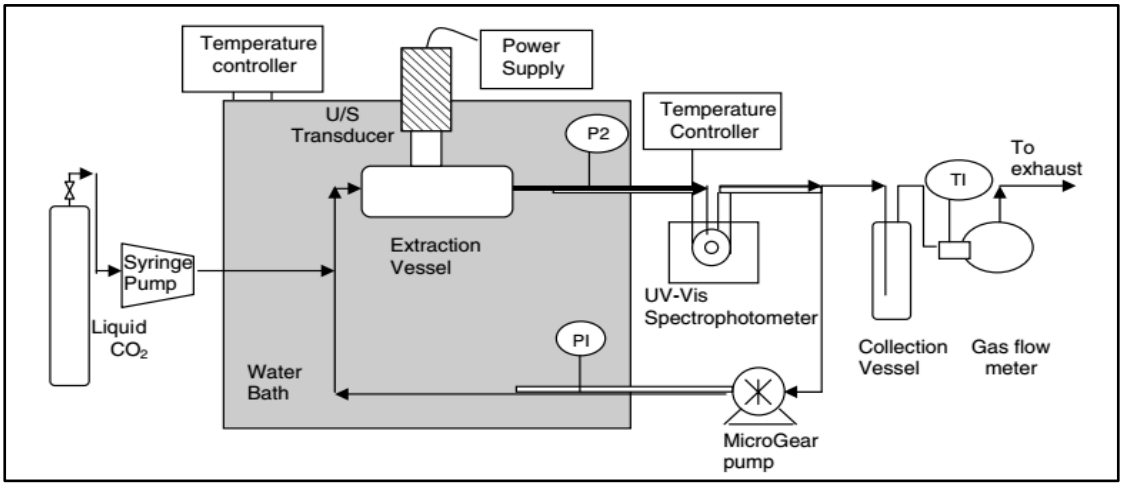

Figure 1.9: Process flow sheet for supercritical CO_2 extraction combined with ultrasounds (Balachandran et al., 2006)

1.3 Glycerol: production, properties and applications

Natural glycerol is the main by-product of two chemical procedures, saponification, and transesterification. The crude glycerol produced contains water, excess alcohol, salt, and fatty acid residues. Thus, it cannot be used in pharmaceutical and food applications without refining. The refining process is complicated and expensive while the price of clean glycerol is low due to the low demand, as such issues of economic viability are rising (Quispe et al., 2013)

Glycerol fulfills certain environmental, economic criteria that confirm its characterization as a "green" solvent. Glycerol in its pure form is a colorless, odorless, sweet, transparent viscous liquid. It contains three hydroxyl groups and as such, is a polar protic solvent. Its dielectric constant is 42.5 (at 25^0C) which is almost half of water (78.5). Glycerol is miscible with water and short-chain alcohols and immiscible with non-polar solvents like ethers and hydrocarbons. At the same time, it can dissolve inorganic salts, bases, acids, etc., providing an ideal environment for reactions and simple removal of their products with liquid-liquid extraction. The boiling point of glycerol is 290 ^{0}C, thus it allows the use of high temperatures increasing the reaction rate. Meanwhile, the removal of more volatile products is feasible through distillation. Glycerol has a clear advantage in comparison to other organic solvents since it is non-toxic (LD50=126000mg/kg), biodegradable and non-flammable, corroborating its safe and environment-friendly character (Gu & Jérôme, 2010; Christoph et al., 2006).

Glycerol is presently used in more than 2000 different applications, and this is due to its unique combination of physical and chemical properties **(Figure 1.10)** (Tan et al., 2013)**.**

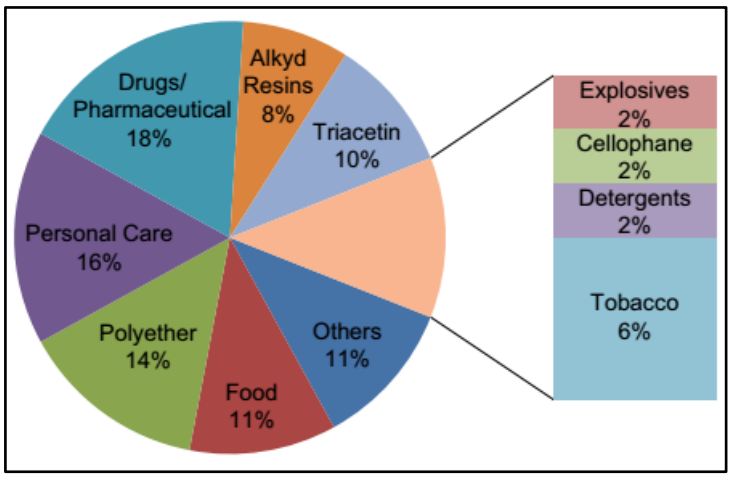

Figure 1.10: Glycerol industrial applications (Tan et al., 2013)

Glycerol traditionally has been used in the food industry as a humectant, sweetener, and preservative. Indirectly, in the form of monoacylglycerides has been utilized as an emulsifier and stabilizer. Glycerol can be found in many dairy products such as cheese and yogurt and bakery goods like bread, cakes, etc. Alcoholic beverages, sauces, butter

formulations, and candies may contain glycerol. As a solvent and a moistener, glycerine has been used in many pharmaceutical products to dissolve active compounds, to increase viscosity in syrups, to modify texture in drug delivery systems, and also as a laxative for constipation treatment. In the cosmetic sector, glycerol is an ingredient of many personal care formulations. It is used in toothpastes to prevent drying and hardening of the product in the tube. The viscosity and humectant properties of glycerol are put in use for the preparation of creams, lotions, deodorants, and makeup products where smoothness, moisture, and lubrication is needed (Mota et al., 2017; Tan et al., 2013).

Besides the many applications of glycerol in the food, pharmaceutical, and cosmetics industry, glycerol serves as a solvent and a catalyst for many synthesis reactions (Chahdoura et al., 2014). It is often used as the HBD for the synthesis of DESs (Abbott et al., 2011). Many value-added products such as propanediol, citric acid, lactic acid, ethanol, etc. utilize glycerol as their synthetic precursor (Bagheri et al., 2015). In the past few years, efforts have been made for the efficient use of glycerol as a green co-solvent for the extraction of bioactive compounds. Water glycerol mixtures have been employed for the extraction of biomolecules from various agri-food by-products such as olive leaves (Apostolakis et al., 2014), eggplant peels (Philippi et al., 2016), apple peels (Blidi et al., 2015), spent filter coffee (Michail et al., 2016), rice bran (Huang et al., 2019) and grapefruit peels (El Kantar et al., 2019). Water/glycerol mixtures have been also utilized for the extraction of bioactive molecules from medicinal plants, like St.John´s wort (Karakashov et al., 2015) and Artemisia species (Shehata et al., 2015). When glycerol/water mixtures were compared with conventional solvents like ethanol and water (Kantar et al., 2019; Phillipi et al., 2016; Karakashov et a., 2015; Apostolakis et al., 2014) they have been proven equal if not superior extractants. Obstacles such as the high viscosity of glycerol and the low recoverability of the extracted compounds can be overcome by the use of heat and appropriate dilutions while the final extract can be used as it is for the intended application.

1.4 Eutectic solvents: evolution, properties, and applications.

The term “deep eutectic solvent” was introduced by Abbot et al (2003) when his team investigated the properties of choline chloride mixtures with urea. Both starting materials were solids -in room temperature- with high melting points while the mixture was liquid and with a lot lower melting point (Abbott et al., 2003). When Choi et al

(2011) reported the formation of viscous solutions by combining choline chloride with natural starting materials such as carboxylic acids, sugars, and water, a new term was suggested, namely “natural deep eutectic solvents” (NADESs). NADESs is not a new class of DESs but it describes low melting points mixtures that are produced by natural ingredients and are more environmentally benign (Choi et al., 2011)**.** In 2012, Kroon’s research team synthesized eutectic mixtures from natural solid components. They observed, after DCS analysis, that the mixtures didn’t have melting points but instead, they had lower glass transition temperatures than the initial counterparts. As such, a new term was proposed, namely “low-transition- temperature mixtures” (LTTMs), expanding the DESs to include liquids with low glass transitions (Francisco et al., 2013).

For a long time, DESs were considered as an evolving subclass of ionic liquids due to some common advantages that they shared and made them appealing as solvents. The similarities between DESs and ILs involve low vapor pressure, customizability and, both are nonflammable. Meanwhile, the initial DES formulations were mostly combinations of a salt (choline chloride) with a hydrogen bond donor (HBD) fact that denotes the ionic nature of DESs as a common feature with ILs. However, DES can be prepared from non- ionic constituents, and their nature as mixtures is non-ionic. Moreover, DESs are considered greener and more easily customizable than ILs and they are cheaper and easier to prepare from renewable starting materials. Consequently, DESs and ILs now considered as two separate groups of solvents (E. L. Smith et al., 2014; Q. Zhang et al., 2012)

DESs are liquid mixtures of natural components where one is an organic salt serving as a hydrogen bond acceptor (HBA), and the other is a biomolecule like sugars, organic acids, etc, that serves as a hydrogen bond donor (HBD) (Georgantzi et al., 2017)**.** The explanation behind the formation of a liquid from two solid counterparts involved the formation of hydrogen bonds that disrupts the lattice of the initial solid components (**Figure 1.11**) (E. L. Smith et al., 2014)**.** Gilli et al., (2009), suggested a way to predict the hydrogen bond strength between the DES's components by comparing their pKa values, briefly the lowest the difference (ΔpKa) between the HBA and HBD, the highest the tendency to form hydrogen bonds

One of the main advantages of DESs is their tunability. The choice of the initial components and their molar ratio in the mixture are important for the properties of the

final mixture. The nature of the salt (HBA) affects the charge shield and thus the hydrogen bond strength with the HBD. At the same time, the different groups (hydroxyl, carbonyl, carboxylic, etc) of the HBD component present different potencies to form hydrogen bond networks with the salt (Francisco et al., 2013)**.** The polarity of DESs has been found that it is related to the nature of HBD counterpart and it is in the order of organic acids> amino acids> sugars> polyols (J. Chen et al., 2019). Zhang et al. (2012) noticed that choline chloride mixtures with polyols and organic acids were producing DESs with lower freezing points which they were liquid at room temperature (Q. Zhang et al., 2012)**.** The presence of water also influences several properties of the DES such as polarity, density, viscosity, and water activity (Dai et al., 2015). Although, water is forming hydrogen bonds with the DES components and might interrupt the network formation between them, in minute quantities water facilitates the hydrogen-bonding network formation (Liu et al., 2018)**.** The addition of water is another way to overcome the disadvantage of the high viscosity of most of the DESs, and together with high working temperatures, to increase the extraction efficiency of the solvent (Chen et al., 2019)

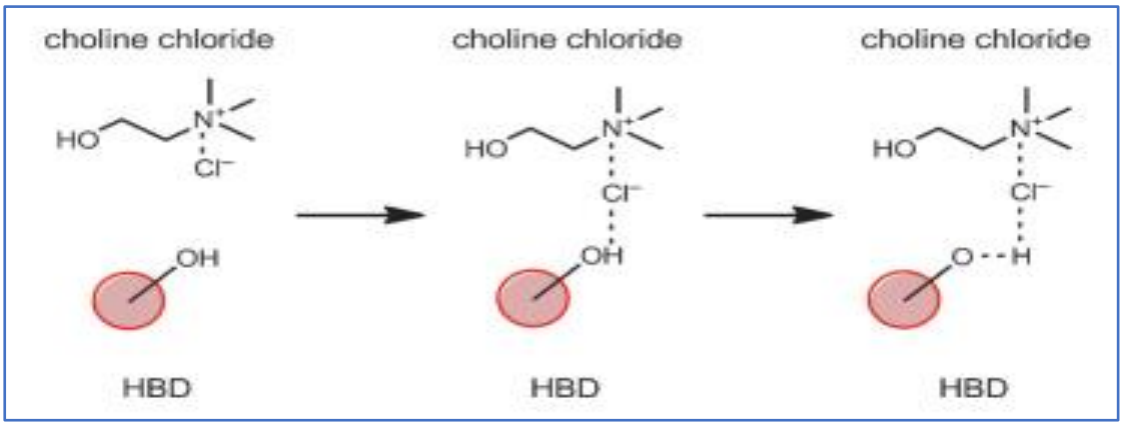

Figure 1.11: Interaction of a HBD with the quaternary ammonium salt (Francisco et al., 2013)

The solubilizing properties of DES and their eco-friendly nature compared to organic solvents made them appealing reaction media. Eutectic mixtures have been employed for the synthesis of organic compounds (Papadopoulou et al., 2016; García-Álvarez, 2015) the synthesis of inorganic compounds (Karimi & Eshraghi, 2017) in chemistry of polymers (Jablonský et al., 2019)**.** DESs have been used successfully as catalysts (Ünlü

et al., 2019)**.** The sorption properties of DESs towards gases like CO_2 and SO_2 have been studied (Sheng et al., 2020; Zulkurnai et al., 2017)**.** The solubility of metals into eutectic mixtures was exploited in the field of electrodeposition (Danilov & Protsenko, 2018)**.** The green character of NADESs and their low toxicity have paved the road for their application in the extraction of bioactive compounds and food analysis (J. Chen et al., 2019; Zainal-Abidin et al., 2017)

1.5 Cyclodextrins (CDs)

Cyclodextrins (CDs) is a group of cyclic oligosaccharides derived from the enzymatic degradation of starch. Their history dates back to the end of 19th century when the French chemist Antoine Villiers discovered a new kind of dextrin, named "cellulosine", which derived from the fermentation of starch with an impure strain of *Bacillus amylobacter*. Later on, at the beginning of the 20th century, Franz Schardinger managed to isolate the correct bacillus strain (*Bacillus macerans*) and produce cellulosine in quantities 10-fold than these reported by Villiers. He was the first to describe their properties, their chemistry, and their structure thus he is considered the "founding father" of cyclodextrin chemistry, even though Villiers was the one who discovered them. The name "cyclodextrins" used for the first time from the german chemist Friedrich Cramer on the title of his doctoral thesis in 1949 (Crini, 2014)**.**

CDs consist of D-glucopyranose units connected with α (1-4) glycosidic bonds. In their native form, their cyclic structure comprises 6, 7, or 8 glucose units and they are named α-, β-, γ- cyclodextrins, respectively. β-cyclodextrin is the most widely used since it's the cheapest and the more accessible (Del Valle, 2004)**.** The structure of cyclodextrins resembles of a hollow, truncated cone **(**Σφάλμα! Το αρχείο προέλευσης της αναφοράς δεν βρέθηκε.**)**. The glucose units are linked covalently forming ether bonds between the adjacent 1 and 4 hydroxyl groups. The secondary hydroxyl groups are located in the narrow rim of the cone while the primary in the wide, between the latter groups, hydrogen bonds are formed and maintain the shape of the molecule. The internal surface of the cone shape molecule is hydrophobic while the external is hydrophilic due to the presence of 21 hydroxyl groups (Crini et al., 2018)

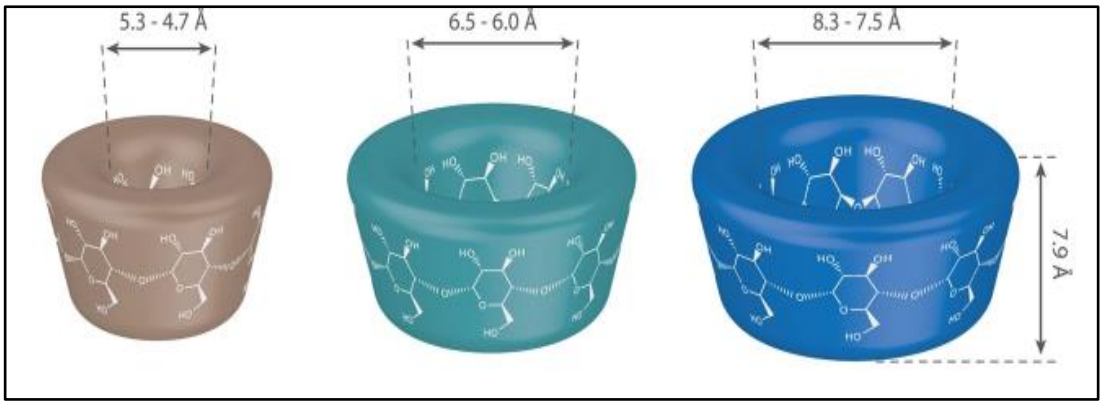

Figure 1.12: Chemical structure and dimensions for α-, β- and γ-cyclodextrin (Crini et al., 2018)

The aqueous solubility of the common CDs is limited because of the formation of hydrogen bonds between the secondary hydroxyl groups of adjacent pyranose molecules and the consequent hindered interaction with the water molecules. Also, the presence of other ingredients (preservatives, surfactants, etc) in the solution decreases the solubility of CDs (Raut et al., 2019)**.**

The main feature of CDs is their ability to form inclusion complexes (ICs) with solute molecules. During this process, a relationship of host-guest is formed where water molecules are substituted in the CDs cavity by the more hydrophobic guest-molecules. The formation of the inclusion complex is related to the three-dimensional relationship between the guest and the host and the local interactions of the CD´s groups and the guest molecule which include hydrophobic interactions and hydrogen bond formation**.** In aqueous solutions, the cyclodextrin and the "guest" molecule coexist in a dynamic equilibrium of recombination-dissociation which is governed by the aforementioned interactions **(Figure 1.13)**. The stoichiometry of the IC represents the number of molecules of "host" and "guest" that interact, in most of the cases the molar ratio is 1:1, however, 1:2 and 2:1 has been reported (Pinho et al, 2014; Astray et al., 2009)**.**

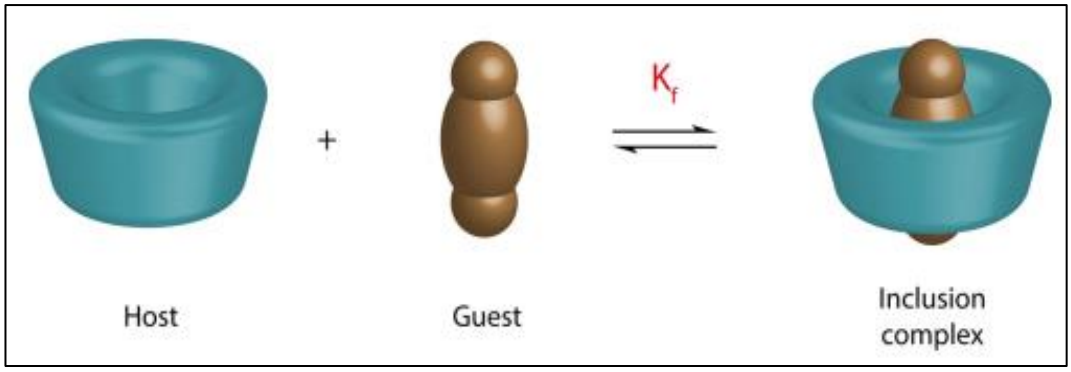

Figure 1.13: Schematic representation of the inclusion phenomena between a cyclodextrin molecule (the host) and a solute (the guest) to form solute–cyclodextrin complexes, Kf is the equilibrium constant (Crini et al., 2018)

Among the native cyclodextrins, α-CD is not an efficient encapsulant because of the small cavity size and γ-CD is expensive. β-CD is cheaper and more widely used but its limited solubility and nephrotoxicity restrain its use in many applications. As such, many chemically modified β-CD derivatives have been synthesized to address solubility, safety, and stability issues (Challa et al., 2005)**.** To date, more than 11000 cyclodextrin derivatives have been reported in the literature. The vast number of cyclodextrins can be justified considering the large number of active hydroxyl groups that are present in the structure of CDs. Hydroxyl groups are good nucleophiles due to the presence of non-bonding electron, thus are prone to electrophilic attacks. The active hydroxyl groups in the molecule of a CD are these in 2, 3, and 6 positions of the pyranose unit. Consequently, there are numerous possibilities for substitution reactions at these sites. There is a degree of selectivity considering that these groups have some differences. The 2 and 3 hydroxyl groups are secondary while the 6 is primary, thus more accessible. The 2 is more acidic and by regulating the pH, selectivity can be achieved. From the previous is obvious that by controlling the reaction conditions (excess of reagent, solvent, etc), products with different degrees and sites of substitution can be derived (Řezanka, 2019)

The encapsulation ability of cyclodextrins has been utilized by the pharmaceutical sector to address problems of low solubility, stability, and bioavailability of the encapsulated drug, as such many papers are discussing the potential of CDs as drug delivery systems (Jansook et al., 2018; Chilajwar et al.,2014; Laza- Knoerr et al., 2010; Challa et al., 2005)**.** The contribution of cyclodextrin technology to the food industry involves the

protection of active encapsulated molecules from heat, oxygen and light, the masking of unpleasant odors, texture modification (emulsions etc), and the incorporation of active ingredients into food matrices (antioxidants, flavors, vitamins, etc) (Aree, 2019; Favre et al., 2018; Szente & Szejtli, 2004). Masking unpleasant smells, control release of aroma substances, and protecting of sensitive ingredients are attributes that are useful to the cosmetic and personal care sector (Tarimci, 2011). Cyclodextrins have been used to encapsulate herbicide (Garrido et al., 2014). The ability of CDs to form ICs with pollutants applies to environmental management and remediation (Fenyvesi et al., 2020; Atteia et al., 2013).

1.6 Aims and Objectives

In the past decades, increasing consumer awareness led the public from the passive approach of buying a product or a service to fulfill specific needs, to more active participation in product development. Buying is not anymore, a selfish, individual decision but encompasses a form of a political statement since the act of purchasing itself is a form of support to the companies' practices, business models, and strategies. Nowadays, consumers recognize their right to be informed, to have access to safe products, and to actively participate in the policy-making of companies.

Lately, consumer's tendency towards a healthier lifestyle changed the requirements in the food industry considerably. Now, consumers increasingly believe that food contributes directly to their health, and as such their expectations have been moved towards new, safe, and added-value products. Thus, foods are no more intended to satisfy hunger and cover basic nutritional needs, but also to prevent diseases and promote physical wellbeing. Food companies, functioning in a competitive environment and responding to the public's demand for a better lifestyle, were forced to seek for new bioactive ingredients that would enhance the properties of foods. As such, terms like "functional foods", "fortified foods" and "health claims" soon became an integral part of the food marketing strategy. Similarly, the cosmetic industry had to adapt to the new consumers' demands. Environmental awareness, animal rights, and the need for safer products led industry stakeholders to turn to new, safer organic materials.

The pharmaceutical sector for a long time has been using nature as a template for the synthesis of new drugs. Phytochemicals like natural products are well renowned for their medicinal properties and plenty of research articles have proven their bioactive efficacy.

An important step for the formulation of reinforced products with phytochemical is their efficient removal from the plant matrix, for this purpose, suitable solvents and extraction techniques have to be employed. Until today, the solvents used for this purpose are byproducts of the oil refinery industry and have been blamed for several adverse effects in human health like respiratory impairment, carcinogenesis, and neurotoxicity. Many organic solvents are volatile and react to sunlight resulting in the formation of "ground-level ozone" which is an air pollutant toxic to animals and plants. The environmental impact of traditional solvents and their toxicity is a topic of interest of a new area of chemistry called "Green chemistry". Among others, green chemistry focuses on the efficient use of energy, the use of renewable materials, and the use of environmentally benign substances, including solvents.

Summarizing; consumer awareness towards functional, safe, and environmentally friendly products have led the industry in a search for new ingredients with bioactive properties. Natural products have proved that they can fulfill these requirements but their extraction is heavily dependent on substances detrimental to human health and the environment. As such, following the principles of green chemistry, and utilizing natural compounds, the objectives of the present work are summarized as follows:

1) The synthesis of a new natural deep eutectic solvent (NADES) and the assessment of its extraction efficiency under optimized conditions.

2) The examination of glycerol extraction potential and the influence of ultrasound waves on the extraction procedure.

3) The contribution of β-cyclodextrin, methyl-β-cyclodextrin and hydroxy-propyl-β-cyclodextrin to the extraction capacity of water under optimized conditions.

4) The stability of the deep eutectic extract under three different storing temperatures and accelerated for a certain amount of time. The effect of mode and structure.

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CHAPTER 2. High-performance green extraction of polyphenolic antioxidants from *Salvia fruticosa* using cyclodextrins: Optimization, kinetics and composition

Abstract

S. fruticosa, collectively known as Cretan sage, is a medicinal plant to which a number of bioactivities have been attributed. In spite its importance in nutrition and pharmacy, reports on the extraction of major polyphenols using sustainable processes is particularly limited. In this study, three common cyclodextrins, namely β-cyclodextrin (β-CD), hydroxypropyl β-cyclodextrin (HP-β-CD) and methyl β-cyclodextrin (m-β-CD) were tested as green boosters of aqueous extraction of polyphenols from aerial parts of *S. fruticosa*. To examine simultaneously important extraction parameters, including the concentration of cyclodextrins (C_{CD}), pH and liquid-to-solid ratio ($R_{L/S}$), a Box-Behnken experimental design was chosen, with three central points. The effect of temperature on the extraction yield was also considered, by carrying out kinetics. The results showed that m-β-CD was the most effective extraction booster, providing total polyphenols yields that amounted 98.39 mg gallic acid equivalents per g of dry mass. The kinetic assay demonstrated that extraction was highly effective at 80 ºC, increasing significantly polyphenol yield, but also the antiradical activity and ferric-reducing power of the extracts. It was also proven that extraction with m-β-CD was the least energy-demanding process. Liquid chromatography-tandem mass spectrometry examination revealed that m-β-CD might possess higher affinity for luteolin 7-*O*-glucuronide extraction, but β-CD for rosmarinic acid extraction.

Keywords: antioxidants; cyclodextrins; extraction kinetics; green extraction; polyphenols; Salvia fruticosa.

2.1 Introduction

The biological significance of medicinal plants has triggered the development of a high number of extraction techniques, which aim at the effective recovery of polyphenolic substances. These techniques may involve the use of volatile and toxic solvents, while the extracts obtained may afterwards require several steps of downstream processes for effective solvent removal and extract recovery. On the other hand, contemporary trends in polyphenol extraction, driven by the need for less environmentally aggravating and safer processes, dictate the development of extraction methodologies that would minimize cost, energy consumption and emission of volatile substances (Belawal et al., 2018). On this philosophy, the replacement of conventional extraction media by novel, green and non-toxic ones is imminent.

Applications of CDs have been increasing on annual basis in pharmaceutical, chemical and other disciplines, but most uses are related with food (Astray et al., 2009). The utilization of CDs in food products pertains mainly to stabilization of flavors, solubilization of poorly water-soluble substances, protection of labile additives etc. However, CDs use for extraction of polyphenolic compounds is a state-of-the-art trend, offering unprecedented opportunities in the so-called "green extraction". This is because, although common organic solvents regularly used for polyphenol recovery (e.g. ethanol, ethyl acetate etc.) display excellent potency for polyphenols dissolution and extraction, their use poses serious environmental concerns. Aqueous systems containing CDs may be regarded as green solvents, with a prospect of replacing organic solvents in relevant processes (Cai et al., 2018).

On such a ground, this investigation was performed with the scope of studying the use of various cyclodextrins, namely β-cyclodextrin (β-CD), hydroxypropyl β-cyclodextrin (HP-β-CD) and methyl β-cyclodextrin (m-β-CD), on the extraction of polyphenols from *S. fruticosa*, using aqueous solutions. The investigation was based on experimental design considering critical extraction parameters, such as the CD concentration, the liquid-to-solid ratio and pH. Finally, temperature effects were assayed by carrying out kinetics, while selectivity issues were checked with liquid chromatography-mass spectrometry analyses.

2.2 Materials and methods

2.2.1 Chemicals

β-Cyclodextrin, hydroxypropyl β-cyclodextrin and methyl β-cyclodextrin were from Sigma–Aldrich (St. Louis, MO, U.S.A.). Ethanol (99.8%) was from Acros Organics (Geel, Belgium). Anhydrous sodium carbonate was from Carlo Erba Reactifs (Val de Reuil, France). 2,4,6-Tripyridyl-*s*-triazine (TPTZ, 99%), Folin-Ciocalteu reagent and ferric chloride hexahydrate were from Fluka (Steinheim, Germany). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), citric acid, gallic acid and ascorbic acid were from Aldrich (Steinheim, Germany). Solvents used for chromatography were HPLC grade.

2.2.2 Plant material

Cretan sage (*Salvia fruticosa*, Lamiaceae) was purchased from a local store (Chania, Crete, Greece) of certified botanicals. The plant material was delivered dried in air-tight, plastic bag packaging and upon receipt it was stored in a dark and dry chamber for no longer than a week. The material was pulverised using a table domestic mill (Tristar, Tilburg, The Netherlands) to provide a powder with average particle diameter of 1.28 mm. This powder was used for all examinations.

2.2.3 Batch stirred-tank solid-liquid extraction

The powdered plant material was extracted with aqueous solvents, which contained 1% (w/v) citric acid, adjusted to the desired pH, and various amounts of either β-CD, HP-β-CD, or m-β-CD. The pH, as well as the exact amount of each cyclodextrin and powdered material were defined by the experimental design (see paragraph 2.4). CDs were incorporated into the aqueous solutions prior to extractions. The extractions were performed under magnetic stirring set at 400 rpm, at ambient temperature (22±1 ºC), at a final volume of 25 mL, in a screw-cap glass vial, for 180 min. After each extraction, samples were centrifuged at 10,000×*g* and the clear supernatant was used for all analyses.

2.2.4 Experimental design

The purpose of the investigation was the effect of cyclodextrin (β-CD, HP-β-CD, m-β-CD) concentration (C_{CD}), pH and liquid-to-solid ratio ($R_{L/S}$) on the performance of aqueous extraction of polyphenols from *S. fruticosa*. To accomplish this, a response surface methodology was set up, using Box-Behnken experimental design with three

central points, which allows estimation of the first- and second-order coefficients of the mathematical model with high reliability (Bezerra, et al, 2008). Yield in total polyphenols (Y_{TP}) was selected as the screening response and the three variables chosen (C_{CD}, pH, $R_{L/S}$) were coded between -1 (lower limit) and 1 (upper limit), as follows:

$$x_i = \frac{X_i - X_0}{\Delta X_i},\ i = 1, 2, 3 \qquad (2\text{-}1)$$

Terms x_i and X_i are the dimensionless and the actual value of the independent variable *i*, respectively. X_0 represents the actual value of the independent variable *i* at the central point of the design, and ΔX_i the step change of X_i, which corresponds to a unit change of the dimensionless value (**Table 2.1**). The ranges used for the independent variables were chosen on the basis of critical evaluation of literature data. ANOVA was performed to acquire model significance, the significance for each polynomial coefficient, and the model coefficient R^2. Based on this analysis, insignificant dependent terms ($p > 0.05$) were not considered in the mathematical equations (models). The desirability function was used to estimate the optimal extraction conditions for maximizing Y_{TP} and visualization of the effect of the independent variables on Y_{TP} was delivered as three-dimensional response surface plots. The models were validated by carrying out experiments under optimal extraction conditions and comparing the values predicted by each model with the experimental ones.

Table 2.1: Actual and coded levels of the process variables considered for the experimental design.

Independent variables	**Code units**	**Coded variable level**		
		-1	**0**	**1**
C_{CD} (%, w/v)	X_1	0.60	1.00	1.40
$R_{L/S}$ (mL g^{-1})	X_2	20	60	100
pH	X_3	3	5	7

2.2.5 Determination of total polyphenols (TP)

A protocol reported elsewhere was used (Karakashov et al., 2015). Aliquot of 0.5 mL of extract was mixed with an equal volume of methanol containing 1% (w/v) trichloroacetic acid in a 1.5-mL Eppendorf tube. Volume of 0.02 mL of this mixture was then combined with 0.78 mL distilled water and 0.05 mL Folin-Ciocalteu reagent. After a 2-min incubation at room temperature, 0.15 mL Na_2CO_3 solution (20% w/v) was added and samples were left to react for 60 min, in the dark. The absorbance at 740 nm was then obtained, using appropriate blank, and the total polyphenol concentration (C_{TP}, mg L^{-1}) was calculated from a calibration curve, constructed with gallic acid as standard. Yield in total polyphenols (Y_{TP}) was estimated by the following equation and expressed as mg gallic acid equivalents (GAE) g^{-1} dry mass (dm):

$$Y_{TP}\ (\text{mg GAE g}^{-1}) = \frac{C_{TP} \times V}{dm} \qquad (2\text{-}2)$$

Where V is the extraction volume (in L) and dm the dry mass of the solid material (in g).

2.2.6 Determination of the antiradical activity (AAR)

For A_{AR} determination, the DPPH assay was used (Karakashov et al., 2015). All samples were diluted 1:20 with methanol prior to analysis. Volume of 0.025 mL of diluted sample was mixed with 0.975 mL of DPPH solution (100 µM in methanol) and the absorbance was immediately recorded at 515 nm ($A_{515(i)}$). The mixture was allowed to react for exactly 30 min and then measurement of absorbance at 515 nm was repeated ($A_{515(f)}$). A_{AR} was calculated as follows:

$$A_{AR} = \frac{C_{DPPH}}{C_{TP}} \times \left(1 - \frac{A_{515(f)}}{A_{515(i)}}\right) \times Y_{TP} \qquad (2\text{-}3)$$

C_{DPPH} and C_{TP} correspond to the DPPH concentration (µM) and total polyphenol concentration (mg L^{-1}) in the reaction mixture, $A_{515(f)}$ the A_{515} at t = 30 min, $A_{515(i)}$ the A_{515} at t = 0 and Y_{TP} is the total polyphenol extraction yield (mg g^{-1}) of the extract. A_{AR} was expressed as µmol DPPH g^{-1} dm.

2.2.7 Determination of the ferric-reducing power (P_R)

P_R of the extracts was assayed as described previously (Karakashov et al., 2015). In a 1.5-mL Eppendorf tube, 0.05 mL of extract, diluted 1:20 with methanol, was mixed with 0.05 mL $FeCL_3$ solution (4 mM in 0.05 M HCl) and the mixture was incubated for 30 min in a thermostated water bath, set at 37 ºC. After incubation, 0.9 mL TPTZ solution (1 mM in 0.05 M HCl) was added, and the absorbance at 620 nm was red after exactly 10 min. P_R was determined from an ascorbic acid calibration curve (50 – 300 µM) and expressed as µM ascorbic acid equivalents (AAE) g^{-1} dm.

2.2.8 High-performance liquid chromatography (HPLC)

The equipment was a FinniganMAT P4000 pump, coupled with a UV6000LP diode array detector (Thermo Scientific, Waltham, MA, U.S.A.). Chromatography was performed on a Superspher RP-18 column, 125 mm × 2 mm, 4 µm, at 40 ºC, maintained at 40 ºC, with a 10-µL injection loop. The eluents were (A) 2.5% acetic acid and (B) methanol. The flow rate was 0.3 mL min^{-1}, and the elution program used was: 0 min, 100%A; 22 min, 65% A; 32 min, 65% A; 60 min, 0% A; 65 min, 0% A.

2.2.9 Liquid chromatography-tandem mass spectrometry (LC/MS/MS)

The chromatograph was a TSQ Quantum Access LC/MS/MS, with a Surveyor pump (Thermo Scientific, Walltham, MA, U.S.A.), interfaced by XCalibur 2.1, TSQ 2.1 software. Analyses were carried out on a Superspher RP-18 column, 125 mm × 2 mm, 4 µm, at 40 ºC, with 10 µL injection volume and a flow rate of 0.3 mL min^{-1}. The column was maintained at 40 ºC. Eluents and elution program were as described above. Mass spectra were acquired in negative ionization mode, with the following settings: sheath gas pressure, 30 mTorr; capillary temperature, 300 ºC; collision pressure at 1.5 mTorr; auxiliary gas pressure, 15 mTorr. Quantification was accomplished with external standard methodology, using a luteolin 7-*O*-glucoside (5 – 1500 µg L^{-1}, R^2 = 0.9982) and a rosmarinic acid (50 – 3000 µg L^{-1}, R^2 = 0.9985) calibration curve. The standards were dissolved in HPLC grade methanol and stored at – 17 ºC.

2.2.10 Statistical analysis

All extraction procedures were repeated at least twice, and all determinations were carried out at least in triplicate. Values were given as means ± standard deviation. All statistics pertaining to the experimental design were provided by JMP™ Pro 13. Linear

and non-linear correlations, as well as curve fittings, were performed with SigmaPlot™ 12.5, at least at a 95% significance level.

2.3 Results and discussion

2.3.1 Optimization of the extraction performance

The process implemented was designed to appraise the effect of three crucial extraction variables, C_{CD}, $R_{L/S}$ and pH, and to identify possible synergistic functions between them. Appraisal of the fitted model and response surface suitability were based on the ANOVA and lack-of-fit test (**Table 2.2**), by considering the closeness of the measured and predicted values (**Table 2.3**). The second-degree polynomial equations (mathematical models), containing only the significant terms, are presented in (**Table 2.4)**, along with the square correlations coefficients (R^2) of the models, which serve as indicators of the total variability around the mean given by the model. Since all total R^2 of the models were equal or higher than 0.97, and the *p* value for lack of fit (assuming a confidence interval of 95%) was highly significant for all models, it can be argued that equations exhibited excellent adjustment to the experimental data. The contour plots constructed on the basis of the models, which are presented on a comparative arrangement in (**Figure 2.1)**, provide an at-a-glance image of the effect of the experimental variables on the response (Y_{TP}), but also illustrate the differences between the three CDs used.

For the extraction with β-CD, C_{CD} was found to exert non-significant effect, suggesting that any shift in C_{CD} within the range tested cannot impact Y_{TP}. The same was observed for HP-β-CD, but for m-β-CD this variable was highly significant (p = 0.0047). This outcome strongly indicated that the nature of the CD used may play a prominent role in the extraction efficiency. On the other hand, no cross term between m-β-CD concentration and either $R_{L/S}$ or pH was significant, showing that combined effects did not occur. Contrary to those, for the extractions performed with any CD, both $R_{L/S}$ and pH were significant.

Quadratic effects of these variables were also significant for the extractions with β-CD and m-β-CD, but for HP-β-CD significant quadratic effect was seen only for pH. Moreover, for HP-β-CD and m-β-CD cross terms of $R_{L/S}$ and pH were significant too, demonstrating that combinations of these two variables may have either negative (HP-

β-CD) or positive (m-β-CD) influence on the extraction yield.

Table 2.2: Statistical data associated with the mathematical models, built using response surface methodology.

Term	Standard error	t Ratio	Probability	Sum of squares	*F* Ratio
β-CD					
Intercept	0.846624	51.50	<.0001*	6.31901	-
C_{CD}	0.518449	1.71	0.1471	239.91451	2.9386
$R_{L/S}$	0.518449	10.56	0.0001*	252.90005	111.5718
pH	0.518449	-10.84	0.0001*	12.14523	117.6107
C_{CD} $R_{L/S}$	0.733198	-2.38	0.0634	0.11560	5.6481
C_{CD} pH	0.733198	-0.23	0.8258	0.65610	0.0538
$R_{L/S}$ pH	0.733198	-0.55	0.6045	1.93408	0.3051
C_{CD} C_{CD}	0.763136	-0.95	0.3865	53.14168	0.8994
$R_{L/S}$ $R_{L/S}$	0.763136	-4.97	0.0042*	82.82608	24.7134
pH pH	0.763136	-6.21	0.0016*	6.31901	38.5181
Lack-of-fit			0.1067	9.972175	8.5298
HP-β-CD					
Intercept	1.05319	44.02	<.0001*	1.88180	-
C_{CD}	0.644945	0.75	0.4859	298.77901	0.5655
$R_{L/S}$	0.644945	9.48	0.0002*	130.81531	89.7874
pH	0.644945	-6.27	0.0015*	3.18623	39.3119
C_{CD} $R_{L/S}$	0.912089	0.98	0.3728	6.94323	0.9575
C_{CD} pH	0.912089	1.44	0.2082	22.94410	2.0865
$R_{L/S}$ pH	0.912089	-2.63	0.0468*	1.93186	6.8950
C_{CD} C_{CD}	0.949333	0.76	0.4805	9.20776	0.5806
$R_{L/S}$ $R_{L/S}$	0.949333	-1.66	0.1571	80.32413	2.7671
pH pH	0.949333	-4.91	0.0044*	1.88180	24.1386
Lack-of-fit			0.1153	15.332875	7.8313
m-β-CD					
Intercept	0.547155	87.27	<.0001*	34.56961	
C_{CD}	0.273577	7.60	0.0047*	195.89960	57.7357
$R_{L/S}$	0.335062	18.09	0.0004*	193.40255	327.1775
pH	0.335062	-17.97	0.0004*	0.18923	323.0072
C_{CD} $R_{L/S}$	0.386897	-0.56	0.6133	3.27610	0.3160
C_{CD} pH	0.386897	2.34	0.1013	17.09663	5.4715
$R_{L/S}$ pH	0.547155	5.34	0.0128*	6.91763	28.5536
C_{CD} C_{CD}	0.47385	3.40	0.0425*	20.80413	11.5533
$R_{L/S}$ $R_{L/S}$	0.47385	-5.89	0.0097*	79.95325	34.7456
pH pH	0.47385	-11.56	0.0014*	34.56961	133.5322
Lack-of-fit			0.8547	0.4840687	0.1844

Table 2.3: Points included in the experimental design, and the corresponding measured and predicted YTP values for the extractions carried out with each of the CDs used.

Design point	Independent variables			Response (Y_{TP}, mg GAE g^{-1} dw)					
	C_{CD} (X_1)	$R_{L/S}$ (X_2)	pH (X_3)	β-CD		HP-β-CD		m-β-CD	
				Measured	Predicted	Measured	Predicted	Measured	Predicted
1	-1	-1	0	30.74	30.97	39.03	39.80	38.16	38.21
2	-1	1	0	46.97	45.41	49.94	50.24	51.03	50.77
3	1	-1	0	34.68	36.24	39.29	38.99	42.54	42.80
4	1	1	0	43.94	43.70	53.77	53.00	54.54	54.49
5	0	-1	-1	35.63	34.81	34.64	35.66	42.68	42.37
6	0	-1	1	25.35	24.38	33.85	32.36	26.50	24.47
7	0	1	-1	45.60	46.57	51.18	52.67	48.64	48.64
8	0	1	1	33.70	34.52	40.81	39.79	42.13	42.44
9	-1	0	-1	42.12	42.70	49.09	47.30	48.47	48.73
10	1	0	-1	45.56	44.82	46.35	45.63	51.03	51.08
11	-1	0	1	31.06	31.80	35.86	36.58	34.93	34.88
12	1	0	1	33.82	33.24	38.39	40.18	41.11	40.85
13	0	0	0	43.93	43.60	45.72	46.36	48.56	47.75
14	0	0	0	43.99	43.60	46.10	46.36	48.50	47.75
15	0	0	0	42.88	43.60	47.27	46.36	46.94	47.75

Table 2.4: Polynomial equations (mathematical models) and statistical parameters calculated after implementation of the experimental design

Cyclodextrin	2nd order polynomial equations	R^2	*p*
β-CD	$43.60 + 5.48X_2 - 5.63X_3 - 3.79X_2^2 - 4.74X_3^2$	0.98	0.0006
HP-β-CD	$46.36 + 6.11X_2 - 4.04X_3 - 2.39X_2X_3 - 4.66X_3^2$	0.97	0.0025
m-β-CD	$47.75 + 2.08X_1 + 6.06X_2 - 6.02X_3 + 2.92X_2X_3 - 2.79X_2^2 - 5.48X_3^2$	1.00	0.0024

Using the desirability function (**Figure 2.2**), it was made possible to estimate the optimal predicted response for each CD tested (**Figure 2.4**). The Y_{TP} achieved with HP-β-CD and m-β-CD were identical and significantly higher than that obtained with β-CD ($p < 0.05$). This finding highlighted the prominent role of the nature of the CD used for the extraction. In support of this are pertinent results on the extraction of olive pomace polyphenols, where HP-β-CD exhibited superior extraction capacity compared with either m-β-CD or γ-CD (Albahari et al., 2018). Data on polyphenol extraction from pomegranate fruit were in the same line (Diamanti et al., 2017), stressing the superiority of HP-β-CD against β-CD as polyphenol extraction booster. In opposition, anthocyanin extraction was more efficient with β-CD rather than HP-β-CD (Y.Zhang et al., 2018). Such discrepancies might emerge from the different encapsulating capacity of the CDs used towards structurally unrelated polyphenolic constituents. Indeed, examinations with pure polyphenols (catechin) demonstrated a more efficient encapsulation with β-CD than HP-β-CD or m-β-CD (S.Ho et al., 2017). Therefore, the higher-performance extraction of *S. fruticosa* polyphenols observed with m-β-CD might reflect the manifestation of such phenomena. Given that the modelling performed revealed significant effect of C_{CD} only for m-β-CD, then it could be postulated that m-β-CD interacted more strongly with *S. fruticosa* polyphenols than β-CD or HP-β-CD within the C_{CD} limits tested.

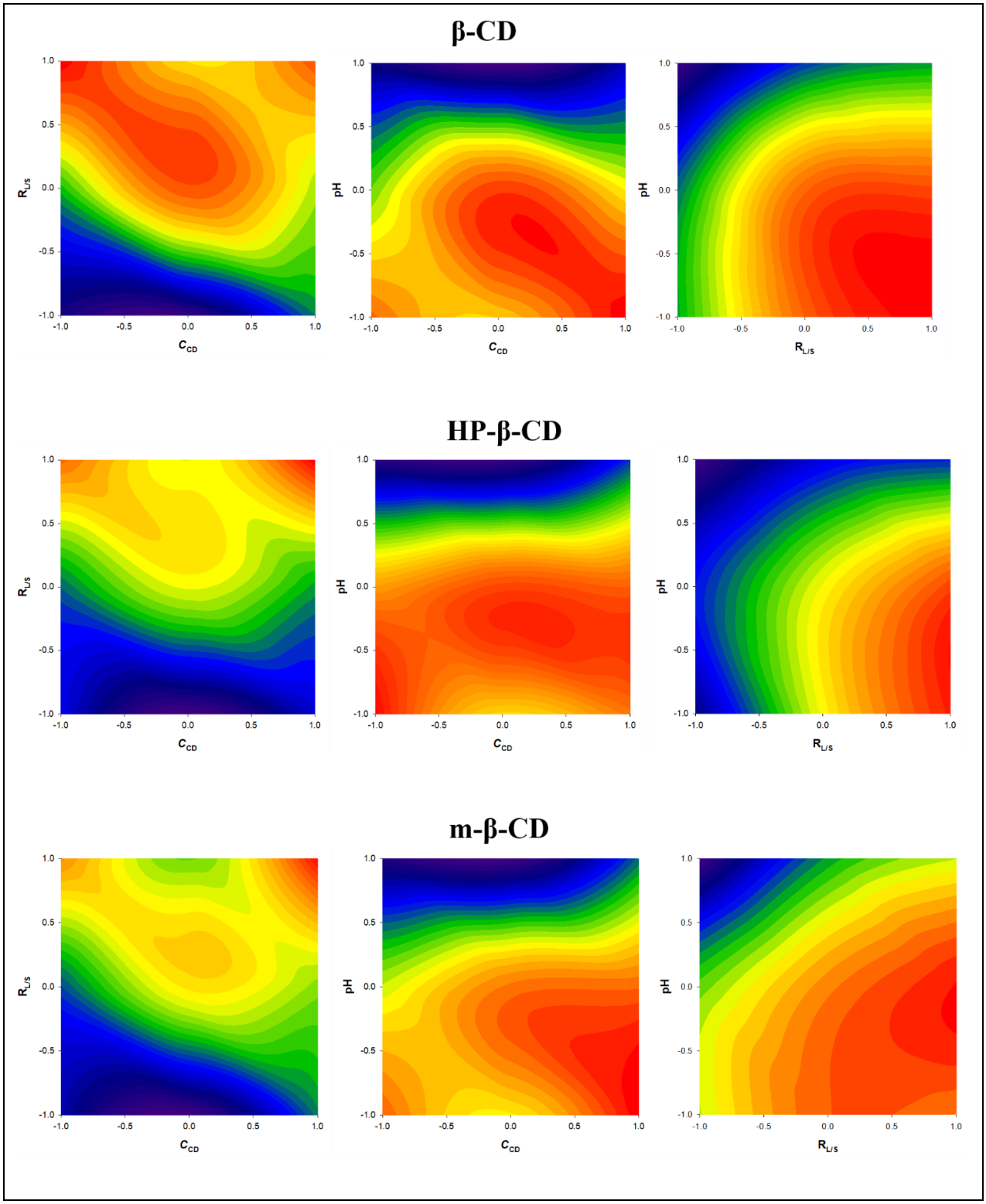

Figure 2.1: Contour plots presenting the effect of simultaneous variation of process variables on the response. Assignments: β-CD, β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin; m-β-CD, methyl β-cyclodextrin.

For all CDs, optimum $R_{L/S}$ varied closely within 93 – 100 mL g^{-1} (**Figure 2.5**). This outcome showed that the influence exerted by $R_{L/S}$ on the extraction performance was not significantly affected by the structure of CD. The magnitude of $R_{L/S}$ is related with the concentration gradient between the liquid phase (extraction medium) and the surface of the solid particle, which is directly involved in mass transfer. If $R_{L/S}$ is below a certain limit, then the equilibria established may not favor fast diffusion of the solute during extraction, due to non-negligible resistance to mass transfer (Rakotondramasy-Rabesiaka et al., 2010). Several examinations on polyphenol extraction from plant material with conventional organic solvents suggested $R_{L/S}$ optima between 81 (Paleologou et al., 2016) to 100 mL g^{-1} (Philippi et al., 2016; Shehata et al., 2015; Blidi et al., 2015). Considering that the average $R_{L/S}$ value in this study was 96 mL g^{-1}, it could be argued that an aqueous medium containing any of the CDs assayed would behave as a common solvent in this regard.

The statistically significant effects of pH revealed by the models for the extraction with any of the CDs assayed, pointed emphatically to the role of the pH in the extraction performance. For all CDs the optimal pH was below 5, which evidenced that extractions were favoured at acidic pH. One possible reason for this might be related with the ionisation of the phenolic hydroxyl groups, which possess weak acidity. Assuming that encapsulation of polyphenols within CD cavity is the main effect that enhances extraction, then polyphenol dissociation would increase their polarity leading to weaker interactions with CD cavity, which is hydrophobic. Since dissociation would increase at higher pH, then it would be likely that suppression of dissociation at pH < 5 would maintain polyphenols in their molecular (non-dissociated) form, hence promoting more powerful polyphenol-CD interactions. In support of such a hypothesis were results on naproxen interactions with β-CD, where increasing pH was demonstrated to provoke instability on the inclusion complex, a fact attributed to reduced affinity of charged drug for the hydrophobic β-CD cavity (Cirri et al., 2006)

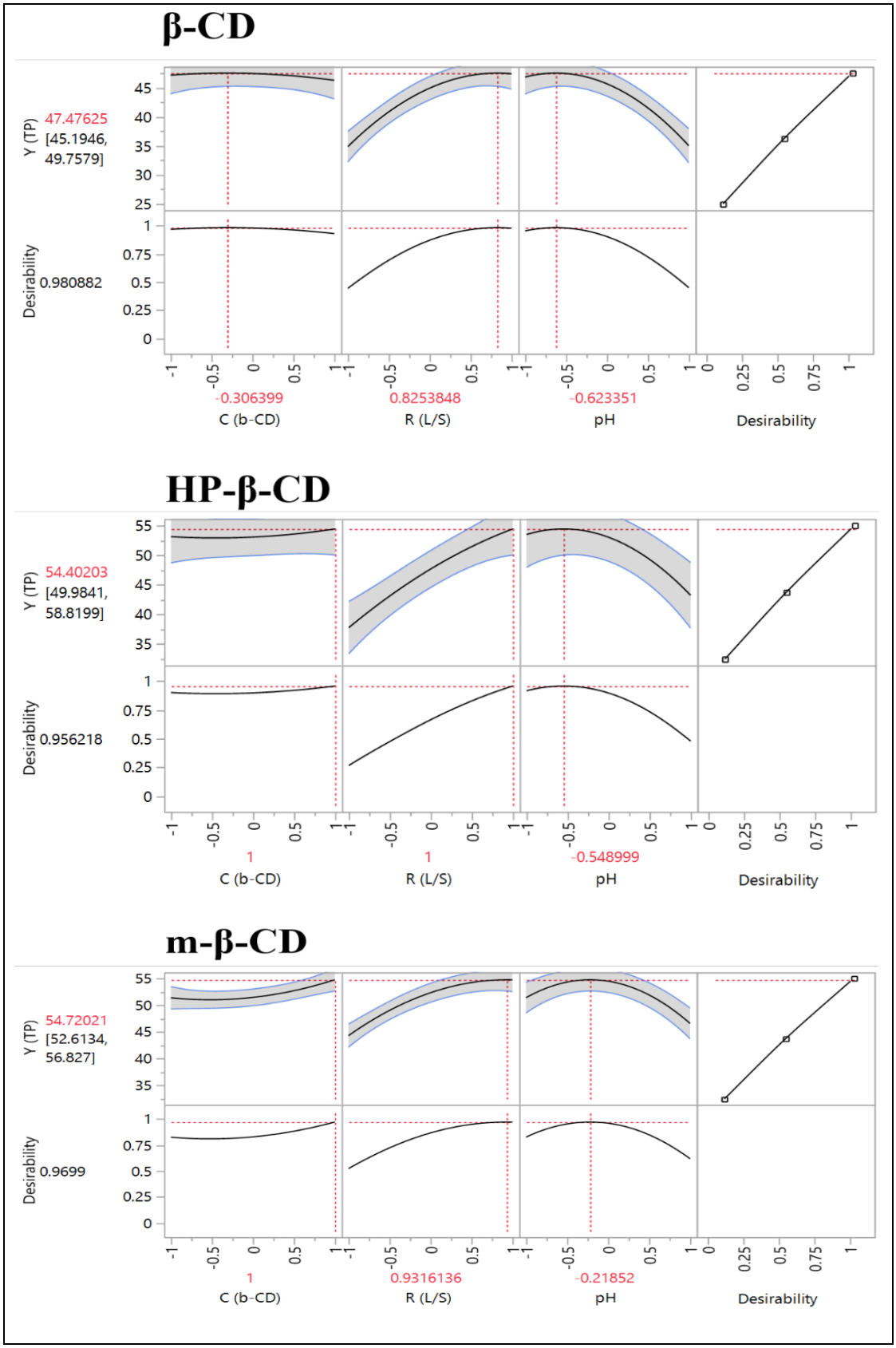

Figure 2.2: Desirability function for each of the CDs tested, displaying optimal conditions and maximum predicted response values. Assignments: β-CD, β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin; m-β-CD, methyl β-cyclodextrin.

Table 2.5: Optimal predicted conditions and maximum predicted values (± sd) for the extraction of *S. fruticosa* by the CDs tested.

Cyclodextrin	Maximum predicted response (Y_{TP}, mg GAE g^{-1} dw)	Optimal conditions		
		C_{CD} (w/v, %)	$R_{L/S}$ (mL g^{-1})	pH
β-CD	47.48±2.29	0.88	93	3.75
HP-β-CD	54.40±4.42	1.40	100	3.90
m-β-CD	54.72±2.11	1.40	94	4.54

Although for several substituted phenolics accurate determination has shown that pK_a may lie well above 7 (Lipatak et al., 2002), for some flavonols such as quercetin, which are frequently encountered in plant tissues, pK_{a1} may vary within 5.06 to 7.36 (Herrero-Martinez et al., 2008). In any case, at pH > 5 even limited dissociation of the most acidic phenolic hydroxyls could occur, provoking significant increase in polyphenol polarity. This issue was also addressed in previous studies on polyphenol extraction with water/ethanol mixtures (Karvela et al., 2009; Kiassos et al., 2009; Mylonaki et al;., 2008), where optimal extraction pH for total polyphenols was always < 5. In these cases, increased extraction yield was ascribed to higher solubility of non-dissociated polyphenols in ethanol-containing solvents and increased polyphenol stability at acidic pH.

Variations in C_{CD} concentration within the limits tested for β-CD and HP-β-CD were shown to exert non-significant impact on Y_{TP}, but it was not clear whether the presence of any CD used could affect Y_{TP}. To examine this, extractions were performed with each CD under optimised conditions, but also with aqueous solutions under the same $R_{L/S}$ and pH, without addition of CD (**Figure 2.5**). In every case, it was demonstrated that addition of CDs provoked significantly higher Y_{TP}, highlighting the importance of the CDs used as aqueous extraction boosters. The highest difference in Y_{TP} was found for m-β-CD (22.06%), followed by HP-β-CD (19.32%) and β-CD (11.28%).

Table 2.6: The effect of each of the CDs tested on the extractability of polyphenols from *S. fruticosa*, compared to equally buffered deionized water, under optimal $R_{L/S}$.

Extraction medium	Y_{TP} (mg GAE g^{-1} dw)	Extraction conditions		
		C_{CD} (w/v, %)	$R_{L/S}$ (mL g^{-1})	pH
β-CD	45.75±1.11	0.88	93	3.75
Buffered dH_2O	40.59±1.01	-	93	3.75
HP-β-CD	50.52±1.19	1.40	100	3.90
Buffered dH_2O	40.76±1.02	-	100	3.90
m-β-CD	54.25±1.35	1.40	94	4.54
Buffered dH_2O	42.28±1.05	-	94	4.54

2.3.2 Extraction kinetics and temperature effects

To assess the effect of temperature on polyphenol recovery, kinetics was traced using all three CDs within a temperature range of 40 to 80 ºC (**Figure 2.3**). The model that could effectively describe the patterns recorded was second-order kinetics (Makris & Kefalas et al., 2015):

$$Y_{TP(t)} = \frac{Y_{TP(s)}^2 kt}{1 + Y_{TP(s)} kt} \quad (2\text{-}4)$$

$Y_{TP(t)}$ and $Y_{TP(s)}$ represent the TP yield at any time *t* and at saturation (equilibrium), respectively. *k* is the second-order extraction rate constant. When *t* approaches 0, the initial extraction rate, *h*, given as $Y_{TP(t)}/t$, is defined as:

$$h = kY_{TP(s)}^2 \quad (2\text{-}5)$$

In **Table 2.7** can be seen the values determined for *k*, $Y_{TP(s)}$ and *h*, using SigmaPlot™ 12.5. For all CDs, the three kinetic parameters exhibited an increase as a response to raising the temperature up to 80 ºC.

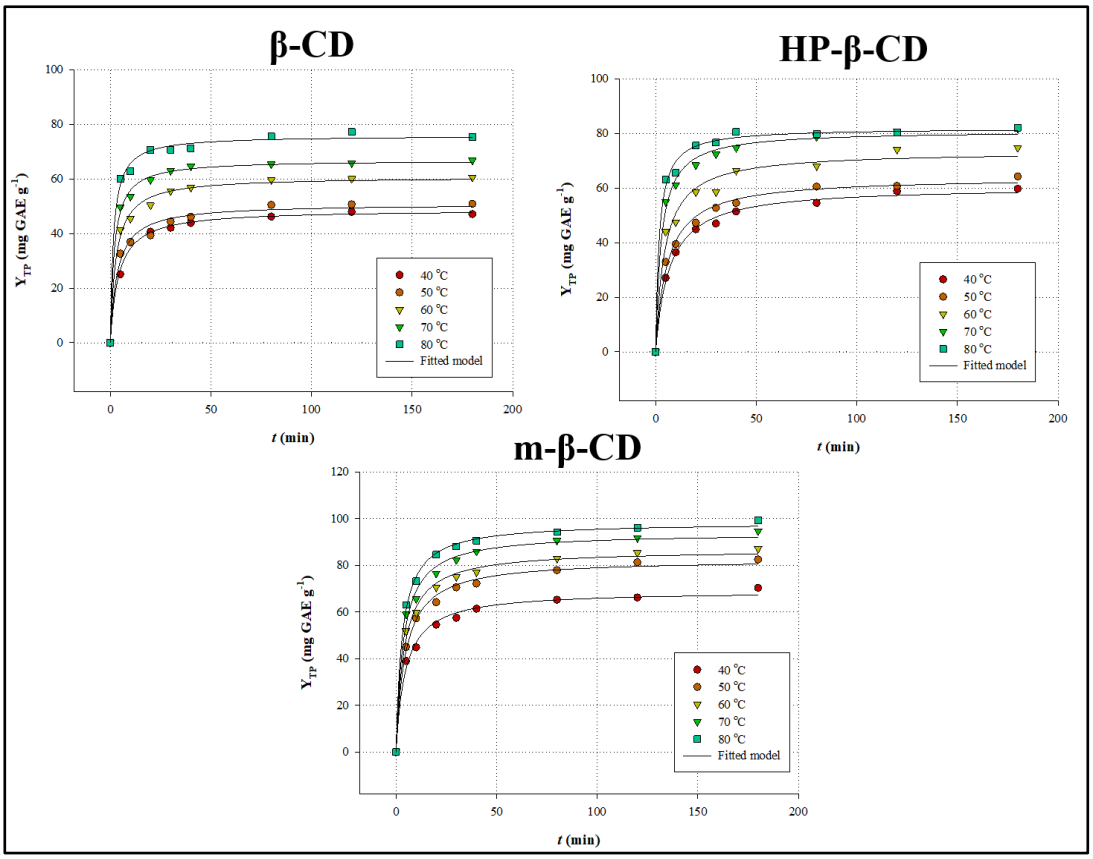

Figure 2.3: Kinetics of polyphenol extraction from *S. fruticosa*, with each of the CDs tested, over a T range varying from 40 to 80 ºC. Extractions were carried out under optimized conditions. Assignments: β-CD, β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin; m-β-CD, methyl β-cyclodextrin.

The highest $Y_{TP(s)}$ was achieved with m-β-CD (98.39 mg GAE g^{-1} dm) at 80 ºC, and it was by only 5.3% lower than that achieved with 60% methanol (**Figure 2.4**). Both β-CD and HP-β-CD were significantly less effective, giving $Y_{TP(s)}$ 75.85 and 81.93 mg GAE g^{-1} dm, respectively. To further assess the impact of temperature, samples obtained at the end of each treatment (180 min) were also assayed for antioxidant activity (**Figure 2.5**). In line with $Y_{TP(s)}$, extracts obtained with m-β-CD exhibited the highest A_{AR} (1112.51 μmol DPPH g^{-1} dm), followed by HP-β-CD (836.17 μmol DPPH g^{-1} dm) and β-CD (824.08 μmol DPPH g^{-1} dm). Results for P_R were in concurrence, giving corresponding values of 241.88, 210.72 and 185.74 μmol AAE g^{-1} dm. This outcome suggested that using m-β-CD, polyphenol-enriched extracts with improved antioxidant characteristics may be produced at 80 ºC.

Table 2.7: Parameters of second-order kinetics, determined for the extraction of TP from *S. fruticosa* with the CDs tested. Extractions were performed under optimal C_{CD}, $R_{L/S}$ and pH.

***T* (°C)**	**Kinetic parameters**		
	***k* (× 10^{-3})** **(g mg^{-1} min^{-1})**	***h*** **(mg g^{-1} min^{-1})**	**$Y_{TP(s)}$** **(mg GAE g^{-1})**
β-CD			
40	4.95	11.76	48.74
50	5.59	14.45	50.86
60	5.83	21.51	60.75
70	7.53	33.70	66.89
80	8.46	48.68	75.85
HP-β-CD			
40	2.43	8.91	60.53
50	2.71	11.09	63.92
60	3.02	16.30	73.41
70	4.40	28.84	80.92
80	6.92	46.47	81.93
m-β-CD			
40	2.55	14.66	68.99
50	2.64	17.99	82.56
60	2.90	21.79	86.71
70	2.95	25.97	93.89
80	3.28	31.76	98.39

The effect of temperature on *k* was better illustrated by establishing correlations between *k* and *T* (**Figure 2.3**). These correlations could be very effectively described using an exponential model (Peleg et al., 2012):

$$k = k_0 + ae^{-bT} \quad (2\text{-}6)$$

Terms *k* and k_0 correspond to the second-order extraction rate and a pre-exponential factor respectively.

In **table 2.8** the parameters k_0, a and b, calculated by SigmaPlot, are given analytically ™ 12.5. Extraction with m-β-CD displayed the lowest b value, which suggested that it was the least affected by temperature, as opposed to the extraction with HP-β-CD. This finding evidenced that m-β-CD provided the most effective and the least energy-

demanding extraction of polyphenols. To ascertain this and obtain a tentative estimation of the barriers required for the extraction with each CD tested, the activation energy was determined as follows (Van-Boekel et al., 1996):

$$ln(\frac{k_{ref}}{k}) = (-\frac{E_a}{R})(\frac{1}{T} - \frac{1}{T_{ref}}) \quad (2\text{-}7)$$

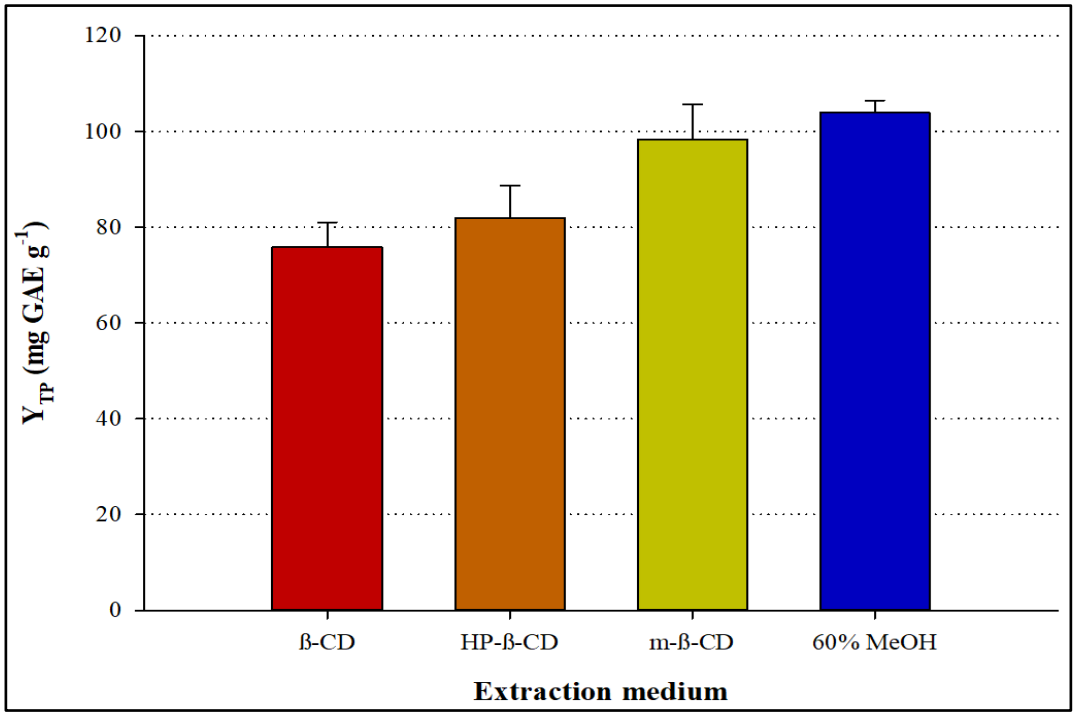

Figure 2.4: Plot showing YTP achieved by using each of the CDs tested, under optimized conditions, at 80 ºC, after 180 min.

Where T_{ref} was chosen as the mean temperature of testing (60 ºC) and T = 40 ºC. k_{ref} and k were the corresponding second-order extraction rate constants. E_a is the activation energy (J mol^{-1}) and R the universal gas constant (8.314 J K^{-1} mol^{-1}). E_a thus estimated for the extraction with β-CD, HP-β-CD and m-β-CD were 7.18, 9.50 and 5.64 kJ mol^{-1}, respectively. This finding did confirm that the extraction with m-β-CD was the least energy-demanding process.

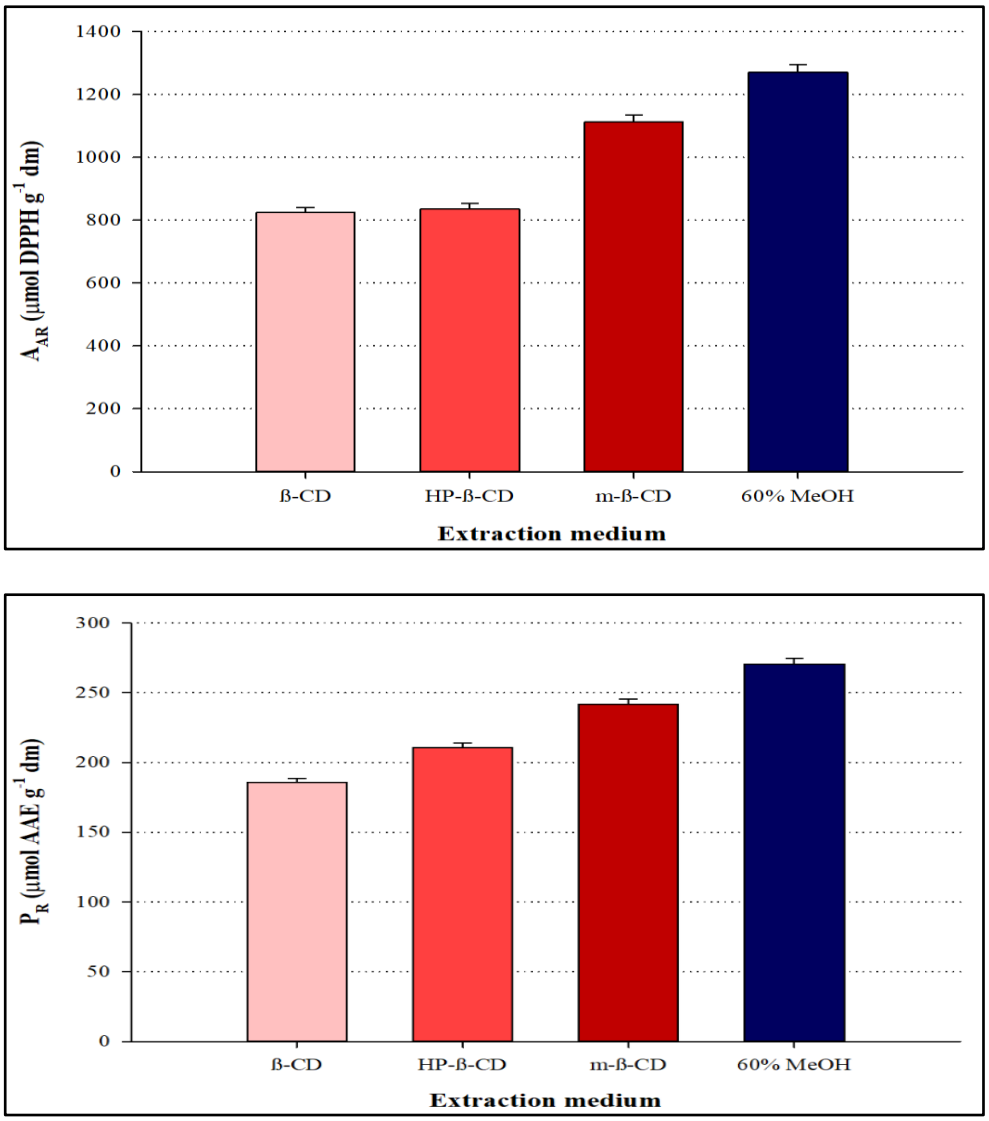

Figure 2.5: Plot showing the AAR and PR of the extracts produced by using each of the CDs tested, under optimized conditions, at 80 ºC, after 180 min.

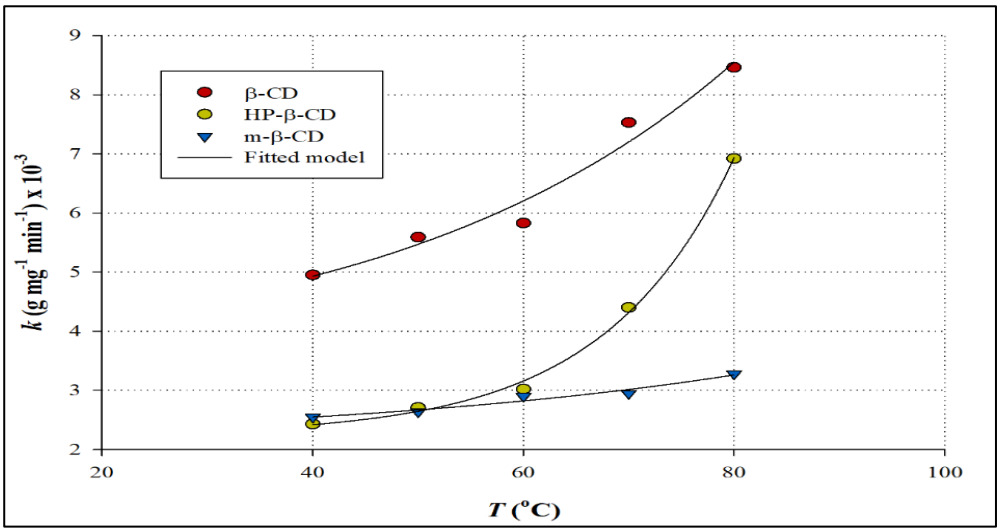

Figure 2.6: Non-linear regression between second-order extraction rates (k) and T.

Table 2.8: Fitting parameter values determined by correlating second-order extraction rates (*k*) with *T*.

CD	Parameter estimates				
	k_0 ($\times 10^{-6}$)	a ($\times 10^{-5}$)	b	R^2	*p*
β-CD	3.409	0.4500	0.0305	0.97	0.0320
HP-β-CD	2.242	0.0068	0.0816	1.00	0.0022
m-β-CD	2.103	0.1727	0.0238	0.96	0.0351

2.3.3 Polyphenolic profile

A chromatogram of a *S. fruticosa* extract, monitored at 350 nm, is given in **Figure 2.7**: HPLC trace recorded at 330 nm, of a *S. fruticosa* extract, obtained with m-β-CD under optimized conditions, at 80 ºC, after 180 min.. The chromatograms corresponding to extracts obtained with either of the cyclodextrins tested did not display any significant difference (data not shown). In total, nine polyphenols could be reliably detected by carrying out LC/MS/MS, but for peak #1 no tentative structure could be proposed (**Table 2.10**). Peaks #2-9 were tentatively identified based on the information provided by previous studies (Koutsoulas et al., 2019; Atwi et al., 2016).

To assess the efficiency of the CDs tested for polyphenol extraction, the two major constituents were considered, luteolin 7-*O*-glucuronide and rosmarinic acid, in order to minimize variations attributed to extraction. As can be seen in **Table 2.9**, extraction with m-β-CD afforded by 7.7% higher luteolin 7-*O*-glucuronide yield compared to β-CD and by 34.4% compared to HP-β-CD. On the other hand, β-CD was by 10.1% more effective compared to m-β-CD and by 13.3% compared to HP-β-CD in extracting rosmarinic acid. This outcome suggested that the structural differences in CDs may account for selectivity towards different polyphenols. In all cases, it was observed that HP-β-CD was the least effective of the three CDs tested, and future studies pertaining to cyclodextrin-aided extraction of polyphenols such as flavonoid glycosides and rosmarinic acid, should consider m-β-CD as the most efficacious extraction booster.

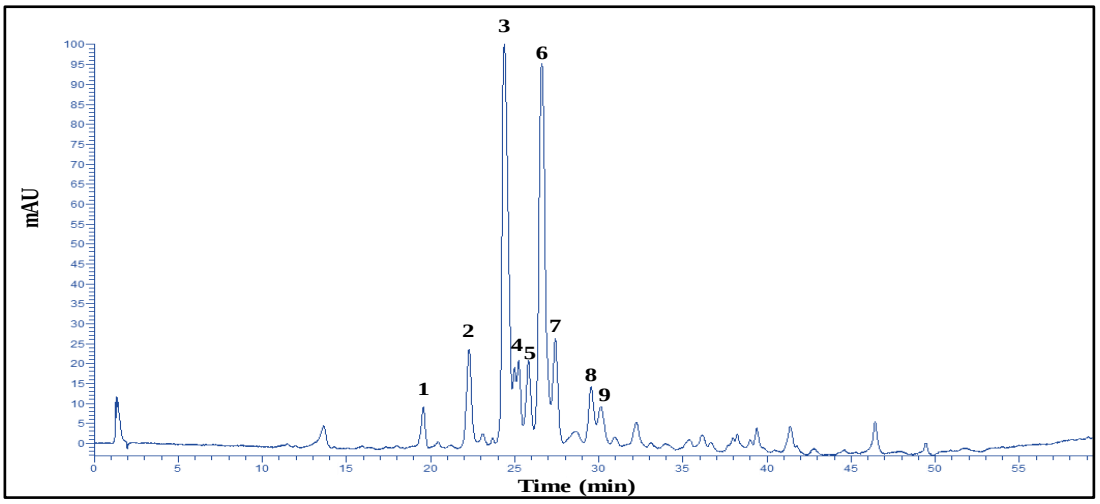

Figure 2.7: HPLC trace recorded at 330 nm, of a *S. fruticosa* extract, obtained with m-β-CD under optimized conditions, at 80 ºC, after 180 min.

Table 2.9: Quantitative data on the recovery of major S. fruticosa polyphenols with the CDs tested, under optimal conditions.

Extract	**Yield (mg g^{-1} dm)**	
	Luteolin 7-*O*-glucuronide	Rosmarinic acid
β-CD	3.35±0.02	7.12±0.00
HP-β-CD	2.38±0.02	6.17±0.10
m-β-CD	3.63±0.03	6.40±0.20

Table 2.10: Spectral information pertaining to polyphenols detected in S. fruticosa extracts, obtained with either CD tested.

No	Rt (min)	UV-vis (λ_{max})	$[M - H]^+$ (m/z)	Other ions (m/z)	Tentative identity
1	19.58	270, 340	593	-	Unknown
2	22.28	280, 344	477	301	6-Hydroxy luteolin 7-*O*-glucoside
3	24.38	256, 352	461	285	Luteolin 7-*O*-glucuronide
4	25.25	258, 348	593	285	Luteolin 7-*O*-rutinoside
5	25.82	270, 352	491	299	6-Methoxyluteolin 7-*O*-glucoside (nepitrin)
6	26.62	246, 316	359	161	Rosmarinic acid
7	27.50	264, 346	445	269	Apigenin 7-*O*-glucuronide
8	29.55	270, 352	475	299	6-Methoxyluteolin derivative
9	30.12	274, 332	461	299, 283	6-Methoxyluteolin derivative

2.4 Conclusions

The current investigation examined in detail the aqueous extraction of bioactive polyphenols from the medicinal plant *S. fruticosa*, aided by the use of three different cyclodextrins. Following process optimization, m-β-CD was proven the most efficient extraction booster, providing extracts with significant polyphenol yield and improved antioxidant characteristics. Extraction kinetics showed that with (i) extraction performance and antioxidant activity may be even more enhanced at 80 ºC and (ii) extraction with m-β-CD was the least energy demanding. LC/MS/MS analyses revealed that luteolin 7-*O*-glucuronide and rosmarinic acid were the predominant polyphenols in the extracts obtained with either CD, and that m-β-CD might exhibit higher affinity for luteolin 7-*O*-glucuronide, and β-CD for rosmarinic acid. The conclusions drawn may be of value in developing green extraction processes for effective polyphenol recovery, not only for *S. fruticosa*, but also other botanical species possessing similar polyphenolic composition. Furthermore, the selectivity issue concerning various CDs should be more thoroughly tested on plant matrices with variable polyphenolic composition, to study the effect of structural features on polyphenol extractability.

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CHAPTER 3. Hydroglycerolic solvent and ultrasonication pretreatment: a green blend for high-efficiency extraction of *Salvia fruticosa* polyphenols

Abstract

Salvia fruticosa (Cretan or Greek sage) is a medicinal plant with significant biological properties, which are largely ascribed to its polyphenolic composition, but there is to-date a scarcity of green and sustainable processes for efficient polyphenol extraction from this plant. The objective of this study was the implementation of an extraction process that would combine a green solvent based on glycerol, a biodiesel industry by-product, and ultrasonication pretreatment. Ultrasonication for 40 min followed by stirred-tank extraction was shown to provide significantly higher total polyphenol yield and extraction kinetics indicated 50 ºC as the most favorable temperature, with the yield being 92 mg GAE per g dry mass. Comparison of this method with a previously developed one that used methyl β-cyclodextrin, revealed that the extracts obtained had almost similar antioxidant activity, and yield in major polyphenols including luteolin 7-*O*-glucuronide and rosmarinic acid was virtually equal. The current process is proposed as a sustainable and effective methodology for the generation of polyphenol-enriched extracts from *S. fruticosa*.

Keywords: antioxidants; extraction kinetics; glycerol; green extraction; polyphenols; *Salvia fruticosa*; ultrasonication

3.1 Introduction

Worldwide, botanicals are used as food but also as folk medicinal agents, based on experience evolved over centuries concerning their nutritional and pharmacological effects. This body of knowledge from the so called "traditional use" has been recognized as a solid ground to support safety and health benefits of botanicals (Anton et al., 2019). To-date, there is an enormous increase of interest concerning bio-products deriving from botanicals with allegedly "functional" properties. Consumer awareness and demand for functional food ingredients and health-promoting supplements have boosted a great development in this area regarding new product design and enabled the launch of a wide spectrum of formulations (Colombo et al., 2020) and cosmetic ingredients (Campa & Baron, 2018).

The development of green processes aiming at producing polyphenol-enriched extracts from botanicals has been of great concern to researchers, and a number of eco-friendly, reproducible, low-cost and low-energy techniques are now acknowledged as more effective alternatives to traditional extraction methodologies (Belwal et al., 2018). However, one of the major ways to comply with the principles of green chemistry is to reduce the use of toxic, volatile organic solvents, and to encourage their replacement by novel, environmentally friendly liquids. In this framework, the selection of an appropriate solvent is of paramount importance to the sustainable character of an extraction method. The ideal candidate should display high extraction efficiency, low or no toxicity, low price, availability, and it should be produced from recyclable resources, as opposed to petroleum-derived solvents (Bubalo et al., 2018; Z.Li et al., 2016).

Although glycerol is well-established sustainable solvent for various chemical processes (Diaz-Alvarez et al.,2011; Y.Gu et al., 2010); Wolfson et al., 2007), its use as a green solvent for effective polyphenol extraction has been introduced only the last six years (Apostolakis et al., 2014). Ever since, several studies have demonstrated glycerol/water mixtures as high-performing extraction media, for polyphenol recovery from various plant matrices (Huamán-Castilla et al., 2020; Eyiz et al., 2020; Lantzouraki et al., 2019; El Kantar et al., 2019; Ciganović et al., 2019; H. Huang et al., 2019; Kyriakidou et al., 2016, D.P.Makris, 2016; Mourtzinos et al., 2016)

This being the case, the current project was undertaken to thoroughly examine the extraction of *S. fruticosa* polyphenolic antioxidants using green glycerol/water mixtures, combined with ultrasonication pretreatment. Major polyphenolic phytochemicals in the optimally produced extracts were tentatively identified with liquid chromatography-diode array-tandem mass spectrometry (LC/MS/MS).

3.2 Materials and methods

3.2.1 Chemicals and reagents

Methyl β-cyclodextrin chlorogenic acid (≥ 95%), luteolin 7-*O*-glucoside and rosmarinic acid (96%) were from Sigma (St. Louis, MO, U.S.A.). Glycerol (99%) and ethanol (99.8%) were from Acros Organics (Geel, Belgium). Aluminium chloride hexahydrate and sodium acetate trihydrate were from Penta (Prague, Czeck Republic). 2,4,6-Tripyridyl-*s*-triazine (TPTZ, 99%), Folin-Ciocalteu reagent and ferric chloride hexahydrate were from Fluka (Steinheim, Germany). Anhydrous sodium carbonate was from Carlo Erba Reactifs (Val de Reuil, France). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), gallic acid, ascorbic acid and rutin (quercetin 3-*O*-rutinoside) were from Aldrich (Steinheim, Germany). The solvents used for chromatographic analyses were HPLC grade.

3.2.2 Plant material – handling and preparation

Salvia fruticosa, also known as *Salvia triloba* (Cretan or Greek sage), was purchased from a local store of certified botanicals (Chania, Greece) and further identified by the Mediterranean Plant Conservation Center (Chania, Greece). The material consisted of dried aerial parts of the plant, and it was received in air-tight plastic packaging. Upon receipt, it was stored in a chamber of low humidity, in the dark, for no longer than a week. Amount of approximately 50 g of material was placed in a domestic blender, ground and then sieved to yield a feed with average particle diameter of 1.28 mm. This feed was transferred into plastic containers, stored at 7 ºC, and used in all procedures.

3.3 Batch stirred-tank solid-liquid extraction

The solvents tested were deionized water, and hydroglycerolic mixtures with glycerol proportion of 20, 40, 60 and 80% (w/v). Exact amount of 1 g of feed was combined with 25 mL of solvent in a 50-mL round-bottom flask, which was then immersed in oil bath at a constant temperature of 50 ºC and stirring at 700 rpm. Temperature regulation and

stirring were provided by a heating magnetic stirrer (VELP Scientifica, NY, USA). Extractions were carried out for 150 min. After extraction, each sample was centrifuged at 10,000×g, for 10 min, and the supernatant was used for all analyses performed afterwards.

3.3.1 Ultrasonication pretreatment

Exact amount of 1 g of feed was mixed with 25 mL of solvent in a 50-mL round-bottom flask, and ultrasonicated in an ultrasonication bath (Sonorex Bandeline, Berlin, Germany) with the following settings: power, 120 W; acoustic energy density, 120 W L^{-1}; frequency, 100 Hz; temperature, 50 ºC. Ultrasonication was performed for 5, 10, 20, 30 and 40 min.

3.3.2 Extraction kinetics and temperature effects

Kinetics was examined by implementing the model previously proposed (Lakka et al., 2019):

$$Y_{TP(t)} = Y_{TP(0)} + \frac{Y_{TP(s)} t}{t_{0.5} + t} \quad (3\text{-}1)$$

$Y_{TP(t)}$ is the yield in total polyphenols at any time t, $Y_{TP(s)}$ the yield in total polyphenols at saturation (equilibrium), $Y_{TP(0)}$ is a fitting parameter and $t_{0.5}$ represents the time at which $Y_{TP(t)} = \frac{Y_{TP(s)}}{2}$. According to this model, the initial extraction rate, h, and the second-order extraction rate, k, are given as:

$$h = \frac{Y_{TP(s)}}{t_{0.5}} \quad (3\text{-}2)$$

$$k = \frac{1}{Y_{TP(s)}\, t_{0.5}} \quad (3\text{-}3)$$

The effect of temperature on k was illustrated by performing non-linear regression between k and T. This correlation could be very effectively described using an exponential model (Peleg et al., 2012):

$$k = k_0 + a e^{-bT} \quad (3\text{-}4)$$

Terms k and k_0 correspond to the second-order extraction rate and a pre-exponential factor. Determination of the activation energy (E_a) of the process was computed as follows (van-Boekel, 2008):

$$ln(\frac{k}{k_{ref}}) = (-\frac{E_a}{R})(\frac{1}{T} - \frac{1}{T_{ref}}) \quad (3\text{-}5)$$

T_{ref} and T are a reference temperature (K) and a temperature at which kinetics was traced, and k_{ref} and k are the corresponding second-order extraction rate constants, R the universal gas constant (8.314 J K^{-1} mol^{-1}) and E_a the activation energy (J mol^{-1}).

3.3.3 Determinations

Total polyphenol analysis was performed using a previously described Folin-Ciocalteu methodology (Karakashov et al., 2015). Yield in total polyphenols (Y_{TP}) was given as mg gallic acid equivalents (GAE) per g dry mass (dm). Likewise, total flavonoids were determined with $CH_3COONa/AlCl_3$ reagent and given as mg rutin equivalents (RtE) per g dm (Kaltsa et al., 2020)The antiradical activity (A_{AR}) and the ferric-reducing power (P_R) were estimated as reported elsewhere (Lakka et al., 2019), and results were expressed as μmol DPPH per g dm and μmol ascorbic acid equivalents (AAE) per g dm, respectively.

3.3.4 Chromatographic determinations

Analyses were performed with a FinniganMAT P4000 pump equipped with a UV6000LP diode array detector (Thermo Scientific, Waltham, MA, U.S.A.), and a TSQ Quantum Access LC/MS/MS, coupled with a Surveyor pump (Thermo Scientific, Walltham, MA, U.S.A.), and controlled by XCalibur 2.1, TSQ 2.1 software. Chromatography was run on a Superspher RP-18 column, 125 mm × 2 mm, 4 μm, maintained at 40 ºC, employing 10-μL injections. The eluents used were (A) 2.5% acetic acid and (B) methanol, at a flow rate of 0.3 mL min^{-1}. The elution program implemented was as follows: 0 min, 100% A; 22 min, 65% A; 32 min, 65% A; 60 min, 0% A; 65 min, 0% A. Mass spectra were acquired with negative ionization, with the following settings: sheath gas pressure 30 mTorr; collision pressure at 1.5 mTorr; capillary temperature 300 ºC; auxiliary gas pressure 15 mTorr. Quantification was carried out with external standard methodology, using a calibration curve of chlorogenic acid (50 – 1500 μg L^{-1}, R^2 = 0.9986), rosmarinic acid (50 – 3000 μg L^{-1}, R^2 = 0.9985) and luteolin 7-*O*-glucoside (5 – 1500 μg L^{-1}, R^2 = 0.9982). Standard solutions were prepared in HPLC grade methanol and stored in the freezer.

3.3.5 Statistical analysis

Two repetitions were performed for each extraction and pretreatment process, and each determination was carried out in triplicate. Values given represent averages ± standard deviation. Linear correlations and kinetic model fitting were accomplished with SigmaPlot™ 12.5 (Systat Software Inc., San Jose, CA, USA). Distribution analysis was done with JMP™ Pro 13 (SAS, Cary, NC, USA)

3.4 Results and discussion

3.4.1 Effect of solvent composition

Earlier examinations on the effect of glycerol/water proportion on polyphenol extraction employed rather low-glycerol mixtures, with glycerol percentage varying from 3.6 (Michail et al., 2016) to 9.3 – 10% (w/v) (Karakashov et al 2015a, Karakashov et al., 2015b; Apostolakis et al., 2014). However, following investigations showed that polyphenol extraction yield may increase linearly from 5% (w/v) and on, the optimum being 20% (w/v) (D.P.Makris, 2016) More thorough, single-factor studies including a wider range of glycerol/water proportions demonstrated that optimum glycerol percentage may lie between 70 (Blidi et al., 2015) and 90% (w/v) (Shehata et al 2015) Optimum levels as high as 90% (w/v) have also been found by implementing response surface methodology (Trasanidou et al, 2016; Katsampa et al., 2015). Yet, significantly lower optimal levels of 20% (w/v) (El Kantar et al., 2019) and (Huamán-Castilla et al., 2020) 32.5% (w/v) have also been reported. On such a ground, testing of the optimum glycerol/water proportion (C_{GL}) was performed over a range varying from 0 (deionized water) to 80% (w/v) glycerol (**Figure 3.1**). Proportions > 80% were not considered, because high-glycerol mixtures are very viscous and particularly problematic to handle. The assay performed indicated that a mixture with C_{GL} of 60% (w/v) provided significantly higher ($p < 0.05$) total polyphenol extraction yield (Y_{TP}), which reached 66.92±1.67 mg GAE g^{-1} dm. Thus, this solution was employed to perform further experimentation.

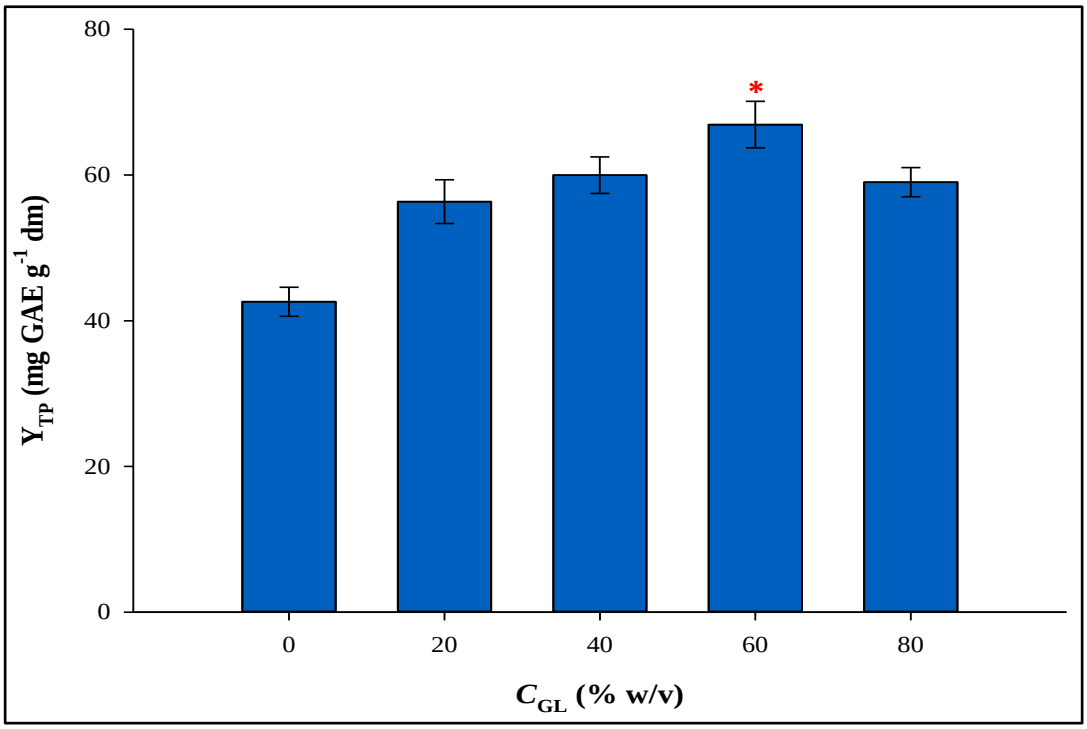

Figure 3.1: Assay performed to identify the optimum concentration of glycerol (CGL) for S. fruticosa polyphenol extraction. Bars indicate standard deviation. Asterisk (*) denotes statistically different value ($p < 0.05$).

3.4.2 Effect of ultrasonication pretreatment

The integration of ultrasonication as a pretreatment stage has been recently appraised, and the ultrasonication time considered was from 5 to 40 min (Lakka et al., 2019; Kaltsa et al., 2020; Trasanidou et al., 2016). On the basis of these data, the ultrasonication effect was tested within this time frame (**Figure 3.2**). As preliminary experiments showed that starting from 25 ºC (room temperature), there may be an increase in temperature up to 45 ºC after 40 min of ultrasonication, the assay temperature was set at 50 ºC, to eliminate variations arising from the ultrasonication effect. Ultrasonication temperature higher than 50 ºC was not preferred, to maximize sonochemical benefit, in line with previous observations (Philippi et al., 2016). It has been proposed that ultrasound-assisted polyphenol extraction was not favored at temperatures higher than 50 ºC, because the collapse of cavitation bubbles generated as a result of ultrasound irradiation, is more effective in low-vapor pressure solvents (such as glycerol/water mixtures) at lower temperatures. The collapse of cavitation bubbles is considered to enhance solute extraction, because there is a release of large amount of energy, as a result of high temperature/high pressure involved in such a process. This in turn may contribute to

disrupting the integrity of the solid particles, provoking increased entrainment of solute in the liquid phase (Chemat & Khan, 2011).

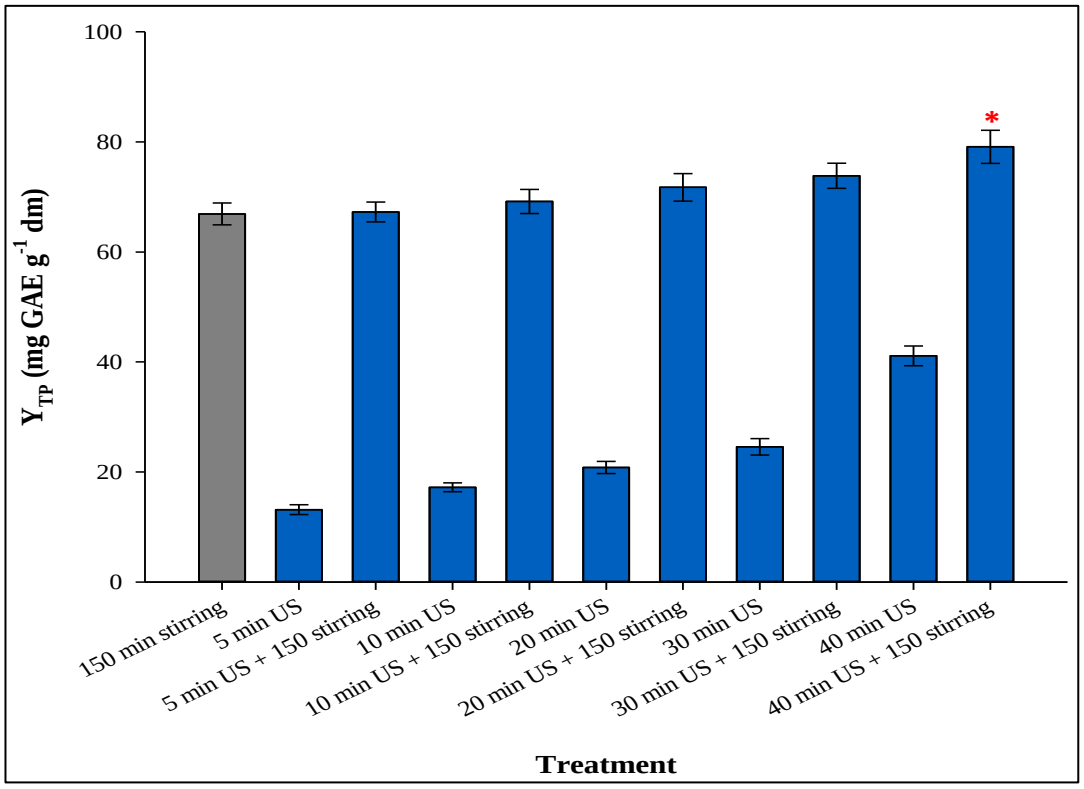

Figure 3.2: The effect of ultrasonication pretreatment on YTP, using 60% (w/v) glycerol/water mixture. Ultrasonication and subsequent stirred-tank extraction were performed at 50 ºC. Bars indicate standard deviation. Asterisk (*) denotes statistically different value ($p < 0.05$).

Changes in Y_{TP} displayed an increasing progression as a function of ultrasonication time, but significantly higher Y_{TP} ($p < 0.05$) was achieved with 40-min ultrasonication pretreatment. The combination of pretreatment and a following stirred-tank extraction afforded a Y_{TP} of 79.12±1.98 mg GAE g^{-1} dm, which was by 15% higher than that attained without pretreatment. This finding stressed emphatically the importance of ultrasonication pretreatment in boosting extraction efficiency. It is to be underlined that mere ultrasonication for 40 min gave a Y_{TP} of only 41.10±1.03 mg GAE g^{-1} dm, which represented approximately just 52% of the Y_{TP} reached by combining ultrasonication pretreatment and stirred-tank extraction. This fact clearly demonstrated that ultrasonication was not effective as a standalone extraction mode. This was in absolute accordance with earlier results from similar studies on grape pomace (Nayak et al., 2018) and elderflowers (Kaltsa et al., 2020).

3.4.3 Extraction kinetics and the effect of temperature

Previous studies showed that polyphenol extraction with hydroglycerolic solvents is significantly affected within a temperature spectrum ranging from 50 to 80 ºC (Trasanidou et al., 2016; Katsampa et al., 2015; Shehata et al., 2015). Thus, kinetics was traced at 50, 60, 70 and 80 ºC to thoroughly investigate the influence of temperature (**Figure 3.3**).

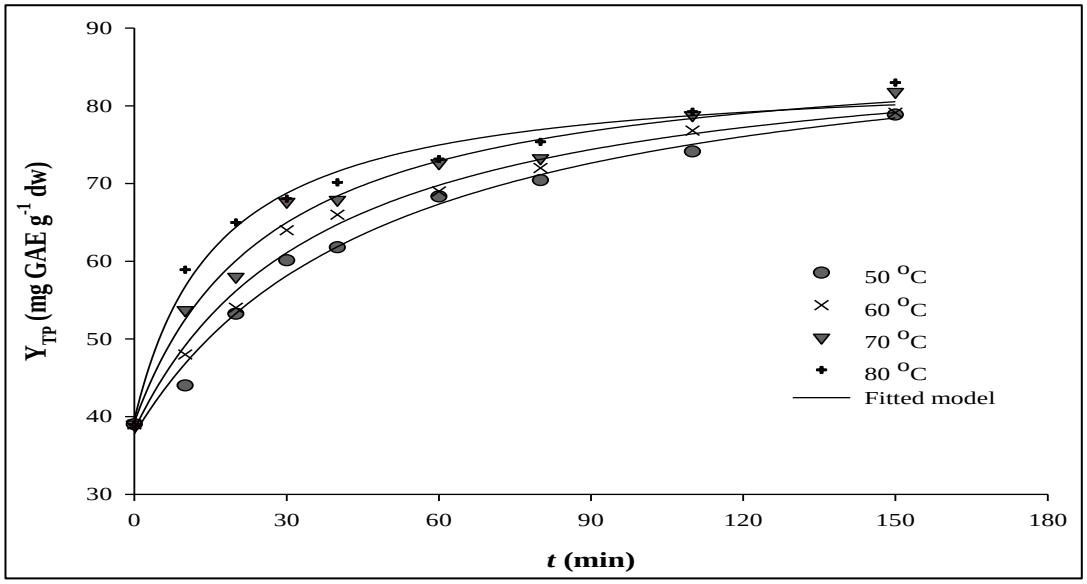

Figure 3.3: Kinetics of S. fruticosa polyphenol extraction, using 60% (w/v) glycerol/water mixture. Samples were pretreated with ultrasounds prior to stirred-tank extraction for 40 min, at 50 ºC.

Switching *T* from 50 to 80 ºC resulted in progressive acceleration of extraction, as indicated by the increase in the second-order extraction rates, *k*, from 0.369 to 1.370 g mg^{-1} min^{-1} (**Table 3.1**). The pattern was similar for the initial extraction rate, *h*, which increased from 1.838 to 5.194 mg g^{-1} min^{-1}. The correlation of *k* with *T* was portrayed by an exponential model, as previously proposed (Peleg et al., 2012), which showed excellent adjustment to the experimental data (**Figure 3.4**). The fitting parameter b equalled 0.0765 and it was significantly higher than 0.0238 determined for aqueous extraction of *S. fruticosa* polyphenols, using methyl β-cyclodextrin (Grigorakis et al., 2020a). This finding suggested that the stirred-tank extraction using hydroglycerolic solvent was more energy-demanding.

Table 3.1: Values of kinetic parameters determined for the extraction of S. fruticosa polyphenols, using 60% (w/v) glycerol/water mixture.

***T* (°C)**	**Kinetic parameters**			
	k ($\times 10^{-3}$) (g mg^{-1} min^{-1})	h (mg g^{-1} min^{-1})	$Y_{TP(s)}$ (mg GAE g^{-1})	$t_{0.5}$ (min)
50	0.369	1.838	92.00	50.06
60	0.528	2.400	89.27	37.19
70	0.768	3.278	87.91	26.82
80	1.370	5.194	84.53	16.27

To ascertain this assumption, the activation energy, E_a, was estimated using the equation (x) and the value found was 47.67 kJ mol^{-1}. This barrier was significantly higher than 5.64 kJ mol^{-1} determined for methyl β-cyclodextrin-assisted extraction (Grigorakis et al., 2020a), which affirmed the higher energy requirement. However, there is an important detail that should be taken into account. In this study, stirred-tank polyphenol extraction was applied after an ultrasonication regime of 40 min, during which a significant amount of readily extractable polyphenols was recovered (**Figure 3.5**). Thus, the E_a determined represented the barrier required to extract the residual and harder-to-extract polyphenols. Such a case has been recently investigated and it was demonstrated that the E_a required to extract polyphenols from plant material after an ultrasonication pretreatment stage was higher than that corresponding to stirred-tank extraction without pretreatment (Lakka et al., 2020)

$Y_{TP(s)}$ displayed a declining trend and while its value was 92.00 mg GAE g^{-1} dm at 50 ºC, it dropped to 84.53 mg GAE g^{-1} dm at 80 ºC. However, distribution analysis indicated that this difference was non-significant ($p < 0.05$). This phenomenon has been previously reported for polyphenol extraction from onion solid wastes with hydroglycerolic mixture and attributed to polyphenol thermal instability (Shehata et al., 2015). In general, increases in T favor higher Y_{TP}, because higher T usually entail higher polyphenol diffusion and solubility (Galanakis et al., 2013; Boussetta et al., 2011).

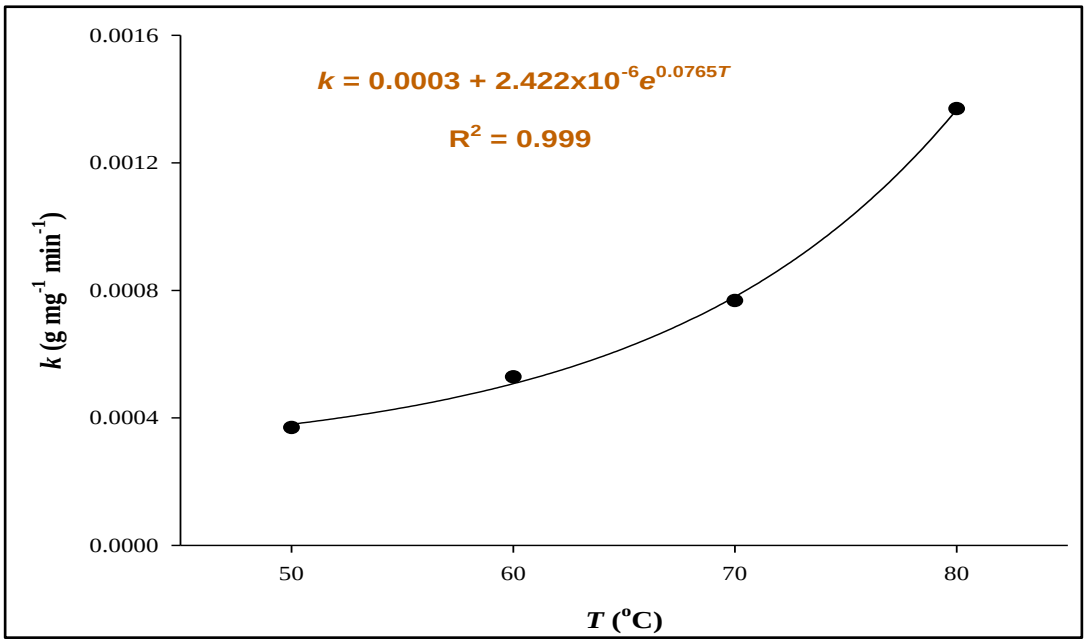

Figure 3.4: Non-linear regression between second-order extraction rate constants, k, and temperature, T.

On the other hand, polyphenols are thermolabile molecules and in several cases *T* higher than 50 ºC did not contribute to attaining increased Y_{TP} (X.Shang et al., 2019; Karageorgou et al., 2017; Khiari et al., 2009). On the other hand, in a previous examination on cyclodextrin-aided aqueous extraction of *S. fruticosa* polyphenols, it was shown that polyphenol extraction yield increased constantly by raising *T* from 40 to 80 ºC (Grigorakis et al., 2020a). Such an effect could be attributed to the protective role of cyclodextrins against thermal degradation of polyphenols, as demonstrated by earlier studies (Mourtzinos et al., 2008).

3.4.4 Antioxidant properties and polyphenolic profile

To test the effectiveness of the method developed, a comparison was carried out with another green method established previously (Grigorakis et al., 2020), based on characteristics pertaining to polyphenol extraction yield and antioxidant activity (**Table 3.2**). Extraction with m-β-CD at 80 ºC was proven more efficient with respect to Y_{TP}, as it afforded 108.14±2.70 mg GAE g^{-1} dm, as opposed to extraction with the hydroglycerolic solvent, which gave by 22.5% lower Y_{TP} (83.86±2.10 mg GAE g^{-1} dm). On the other hand, differences in Y_{TFn} and A_{AR} were marginal and non-significant ($p < 0.05$). On the contrary, the hydroglycerolic extract exhibited significantly higher P_R. The LC/DAD/MS/MS enabled the tentative identification of a series of polyphenolic

phytochemicals (**Figure 3.5**, **Table 3.3**), based on spectral data reported earlier (Grigorakis et al., 2020a; Atwi et al., 2016).

Table 3.2: Comparative evaluation of *S. fruticosa* extracts obtained with 60% (w/v) glycerol/water (GL) and methyl β-cyclodextrin (m-β-CD).

Extract	**Y_{TP}** (mg GAE g^{-1} dm)	**Y_{TFn}** (mg RtE g^{-1} dm)	**A_{AR}** (μmol DPPH g^{-1} dm)	**P_R** (μmol AAE g^{-1} dm)
m-β-CD	108.14±2.70	53.62±1.61	820.93±16.42	590.66±14.77
GL	83.86±2.10	51.46±2.57	81758±8.18	709.12±17.73

In order to better demonstrate the extraction capacity of the hydroglycerolic solvent, three major constituents were considered for quantitative analysis, namely chlorogenic acid, luteolin 7-*O*-glucuronide and rosmarinic acid. Other minor polyphenols that tentatively identified in the extracts were not considered, because they occurred at significantly lower levels and differences in their content might not be indicative for reliably assessing solvent extraction capacity. The results from the quantitative assay are analytically presented in **Table 3.4**. Compared to m-β-CD, extraction with the hydroglycerolic solvent gave by 37.5% higher yield in chlorogenic acid and by 0.57% higher yield in rosmarinic acid, but by 20.8% lower yield in luteolin 7-*O*-glucuronide. Overall, the difference in yield was only 7.4%, indicating that both extracting media performed equally in the recovery of major *S. fruticosa* phytochemicals.

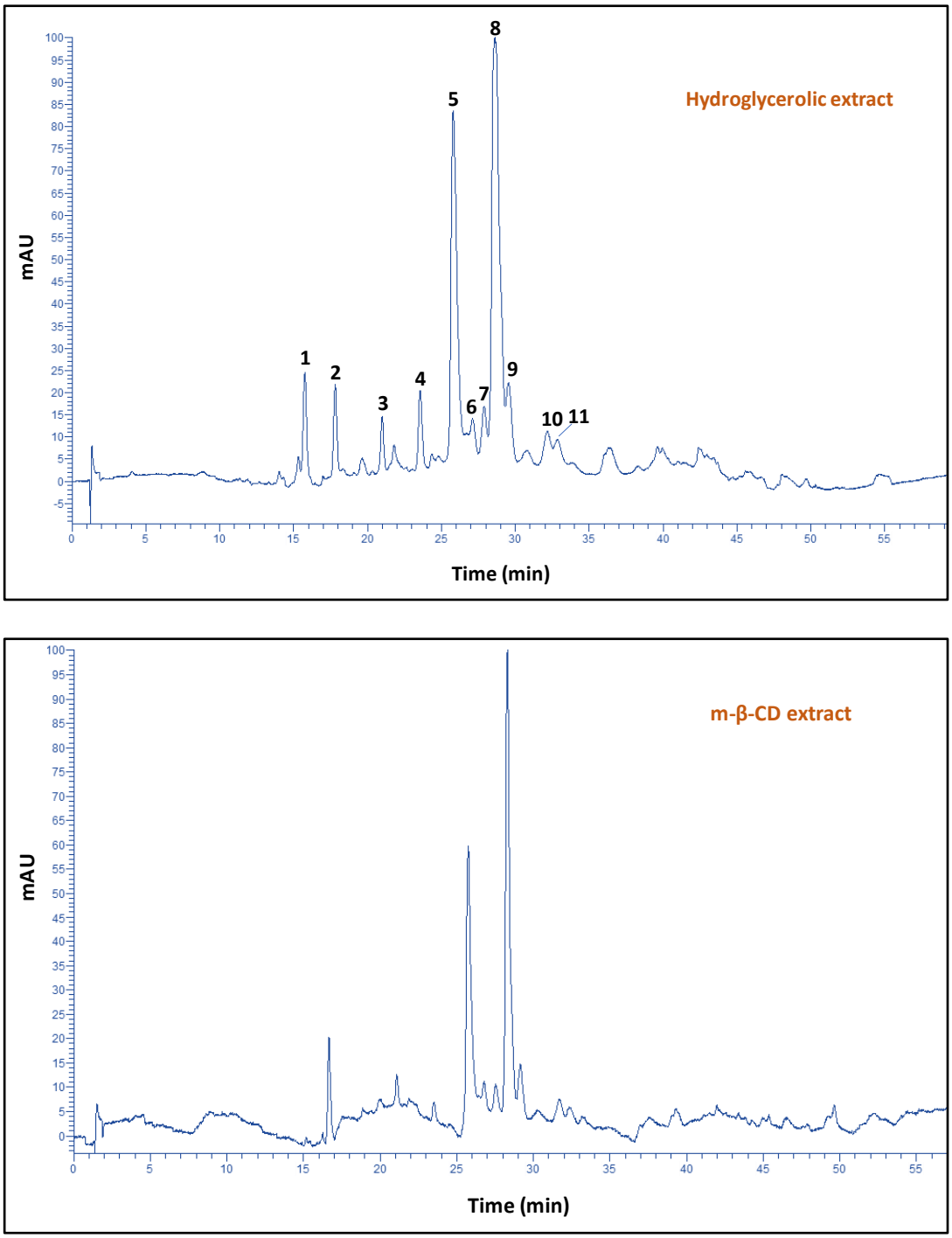

Figure 3.5: Typical HPLC traces of S. fruticosa polyphenol extracts, monitored at 330 nm. Extracts were produced with with 60% (w/v) glycerol/water and methyl β-cyclodextrin (m-β-CD), at 80 ºC. For peak assignment, see Table 3.3

Table 3.3: Spectral attributes of used to tentatively identify major polyphenols in *S. fruticosa* extracts.

Rt (min)	UV-vis (λ_{max})	$[M - H]^+$ (m/z)	Other ions (m/z)	Tentative identity
15.77	246, 318	353	179	Chlorogenic acid
17.40	248, 318	253	-	Unknown
21.00	270, 340	593	-	Unknown
23.57	280, 344	477	301	6-Hydroxy luteolin 7-*O*-glucos
25.78	256, 352	461	285	Luteolin 7-*O*-glucuronide
27.12	258, 348	593	285	Luteolin 7-*O*-rutinoside
27.90	270, 352	491	299	6-Methoxyluteolin7-*O*-glucosi (nepitrin)
28.65	246, 316	359	161	Rosmarinic acid
29.55	264, 346	445	269	Apigenin 7-*O*-glucuronide
32.15	270, 352	475	299	6-Methoxyluteolin derivative
32.82	274, 332	461	299, 283	6-Methoxyluteolin derivative

Table 3.4: Quantitative information on major polyphenols considered to compare S. fruticosa extracts produced with with 60% (w/v) glycerol/water (GL) and methyl β-cyclodextrin (m-β-CD).

Compound	Yield (mg g^{-1} dm) ± sd	
	m-β-CD	GL
Chlorogenic acid	0.15±0.02	0.24±0.05
Luteolin 7-*O*-glucuronide	6.96±1.12	5.51±1.57
Rosmarinic acid	10.57±1.37	10.63±0.98
Sum	17.68	16.38

3.5 Conclusions

The approach attempted with this study aimed at (i) utilizing glycerol, a by-product of the biodiesel industry, as a green and non-volatile solvent, and (ii) integrating ultrasonication pretreatment as a step central to increasing the efficiency of the extraction methodology used. The combination of such a pretreatment with a hydroglycerolic solvent provided a high-efficiency extraction for *S. fruticosa* polyphenols. The kinetics showed that extraction at 50 ºC may be the most favorable, and thus this methodology may also be energy-effective, a fact that significantly adds to the sustainable profile of the process. A prospect of this investigation would be future studies focusing on scale-up and application of hydroglycerolic extracts of *S. fruticosa* as effective food antioxidants/antimicrobials and/or cosmetic constituents. This would pave the way for implementation of the process on industrial scale.

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CHAPTER 4. Batch stirred-tank green extraction of *Salvia fruticosa* polyphenols using newly designed citrate-based deep eutectic solvents and ultrasonication pretreatment

Abstract

A series of citrate salts were tested as hydrogen bond acceptors to synthesize deep eutectic solvents based on lactic acid and glycerol. The DES produced were then screened to identify the highest performing system for the effective extraction of polyphenolic phytochemicals from the medicinal plant *Salvia fruticosa* (Greek sage). The most efficacious DES was the one composed of lactic acid and sodium citrate dibasic, at a molar ratio of 15:1 (LA-SCDB15). Furthermore, for the first time there has been evidence concerning DES pH and extraction efficiency. Using this solvent, a batch, stirred-tank extraction process was developed, by employing ultrasonication pretreatment and response surface methodology. The optimal settings determined were stirring speed 900 rpm, proportion of DES/water 77% (w/v), and ultrasonication time 15 min. By adjusting these optimal settings, the predicted maximum total polyphenol yield was calculated to be 79.93±1.92 mg gallic acid equivalents g^{-1} dry mass. The examination of temperature effects demonstrated that the batch, stirred-tank extraction stage was very energy-efficient, with a barrier of 7.64 kJ mol^{-1}. Comparison of the extraction of *Salvia fruticosa* polyphenols with other green processes previously developed, illustrated the high extraction capacity of LA-SCDB15. The major polyphenols identified in the extracts produced under optimized settings were chlorogenic acid, luteolin 7-*O*-glucuronide and rosmarinic acid.

Keywords: antioxidants; deep eutectic solvents; extraction kinetics; polyphenols; *Salvia fruticosa*; ultrasonication

4.1 Introduction

Deep eutectic solvents (DES) are innovative liquids, composed of low-cost, non-toxic and recyclable materials, which can be naturally occurring compounds (e.g., organic acids and salts, polyols, sugars, etc.). DES are usually composed of a substance functioning as hydrogen bond donor (HBD) and another one as hydrogen bond acceptor (HBA), and their synthesis is straightforward and benign. DES possess features such as absence of flammability water (im)miscibility and low vapor pressure, and these attributes make DES suitable solvents for a spectrum of sustainable applications (Paiva et al., 2014). To-date, by virtue of their unique properties, the use of DES for natural product extraction has been rapidly expanding, and there has been a bewildering number of substances used for DES synthesis.

However, up to now the development of green extraction processes for the production of polyphenol-containing bioactive extracts from *S. fruticosa* based on DES, is inexisted. In such a frame, the current study had as objective the establishment of a green extraction methodology, by blending ultrasonication pretreatment and a highly efficacious DES, selected out of a thorough screening.

4.2 Materials and methods

4.2.1 Chemicals

Chromatography solvents were HPLC grade. L-lactic acid (80%) was obtained from Fisher Scientific (Loughborough, UK). Sodium carbonate, sodium citrate dibasic sesquihydrate (>99%), sodium citrate monobasic (99%), sodium acetate trihydrate, ascorbic acid, rosmarinic acid, luteolin 7-*O*-glucoside, chlorogenic acid and 2,2-diphenylpicrylhydrazyl (DPPH) were from Sigma-Aldrich (Darmstadt, Germany). Sodium citrate tribasic dihydrate (>99%), Folin-Ciacalteu reagent, glycerol (99%) and citric acid and were from Merck (Darmstadt, Germany). Methanol and ethanol were from Honeywell/Riedel-de Haen (Seelze, Germany). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) and iron chloride hexahydrate were from Honeywell/Fluka (Steinheim, Germany).

4.2.2 Plant material

Details regarding plant material source and handling have been described elsewhere (Grigorakis et al., 2020a). In short, dry and powdered *S. fruticosa*, with mean particle size of 1.28 mm, was used in all experiments. The material was from the area of Chania

(Crete, southern Greece) and it composed of the aerial parts of the plant. Plant species was identified by the Mediterranean Plant Conservation Center (Chania, Greece).

4.2.3 Preparation of the DES

To protocol followed for DES synthesis was based on a previously reported one (Jancheva et al., 2017). Precise mass of HBD was mixed with HBA at various molar proportions, and the mixtures were heated at 70 ºC, under continuous stirring at 500 rpm, until the formation of perfectly transparent liquids. This process usually required 60 min, depending on HBD/HBA combination and molar ratio. All DES produced were stored in glass screw-cap vials, at ambient temperature, in the dark, and they were periodically inspected for appearance of crystals over 5 weeks.

4.2.4 Ultrasonication pretreatment

Ultrasonication of samples was applied prior to batch stirred-tank extraction, using an ultrasonication bath (Sonorex Bandeline, Berlin, Germany). The ultrsonication was carried out at ambient temperature, with the following settings: frequency, 100 Hz; power, 120 W; acoustic energy density, 120 W L^{-1}.

4.2.5 Batch stirred-tank extraction process

For the screening process, all DES were tested as 70% (w/v) aqueous mixtures. Control solvents were 60% (v/v) ethanol, 60% (v/v) methanol and deionized water. Extractions were accomplished in a 20-mL glass vial, using 15 mL of each solvent and 0.375 g of plant material, for 150 min. Continuous stirring at 500 rpm and regulation of temperature at 50 ºC were provided by a stirring hot plate (VELP Scientifica, NY, USA). After the extraction, extracts were centrifuged at 10,000×*g* for 10 min, to obtain a clear supernatant used for all determinations.

4.2.6 Design of experiment and response surface methodology

Response surface methodology was implemented through a Box-Behnken design with three central points, to assess the effect of selected process variables on the total polyphenol yield (Y_{TP}, mg GAE g^{-1} dm). The variables considered were the stirring speed (S_S, rpm), the DES/water proportion (C_{DES}, % w/w) and the ultrasonication pretreatment time (t_{US}, min), which were assigned as X_1, X_2 and X_3, respectively. Codification of the variable levels (**Table 4.1**) was done as described in detail elsewhere (Lakka et al., 2019a). Model fitting to the experimental data was evaluated by performing ANOVA

and lack-of-fit analysis, and non-significant dependent terms were excluded from the model (mathematical equation).

Table 4.1: Actual and coded levels of the independent variables selected to set up the experimental design.

Independent variables	**Code units**	**Coded variable level**		
		-1	**0**	**1**
S_S (rpm)	X_1	300	600	900
C_{DES} (%, w/w)	X_2	55	70	85
t_{US} (min)	X_3	5	10	15

4.2.7 Extraction kinetics

The kinetic model employed has been previously reported (Karageorgou et al., 2018). The model is described by the following equation:

$$Y_{TP(t)} = \frac{Y_{TP(s)}t}{t_{0.5}+ t} \quad (4\text{-}1)$$

$Y_{TP(t)}$ and $Y_{TP(s)}$ correspond to the yield in total polyphenols at any time, t, and at saturation (equilibrium). The term $t_{0.5}$ corresponds to the time at which $Y_{TP(t)} = \frac{Y_{TP(s)}}{2}$. The initial extraction rate, h, and the second-order extraction rate, k, can be determined by the following equations:

$$h = \frac{Y_{TP(s)}}{t_{0.5}} \quad (4\text{-}2)$$

$$k = \frac{1}{Y_{TP(s)}\, t_{0.5}} \quad (4\text{-}3)$$

The influence of temperature on k was portrayed by non-linear regression between k and T. Effective description of this correlation could be given by an exponential model, as previously proposed (Peleg et al., 2012):

$$k = k_0 + ae^{-bT} \quad (4\text{-}4)$$

Term k corresponds to the second-order extraction rate and k_0 is a pre-exponential factor. Where a and b are fitting parameters. Estimation of the activation energy (E_a) of the process was calculated as follows (van Boekel et al., 2019):

$$ln(\frac{k}{k_{ref}}) = (-\frac{E_a}{R})(\frac{1}{T} - \frac{1}{T_{ref}}) \quad (4\text{-}5)$$

T_{ref} and T represent a reference temperature (K) and a temperature at which kinetics was traced, k_{ref} and k correspond to the second-order extraction rate constants, R is the universal gas constant (8.314 J K^{-1} mol^{-1}) and E_a the activation energy (J mol^{-1}).

4.2.8 Determinations

Total polyphenol concentration was determined with the Folin-Ciocalteu methodology and yield in total polyphenols was expressed as mg gallic acid equivalents (GAE) per g dry mass (Karakashov et al., 2015). Total flavonoid determination was carried out with the aluminium chloride reagent and results were expressed as mg rutin equivalents (RtE) per g dry mass (Kaltsa et al., 2020a). The antiradical activity (A_{AR}) was estimated with a stoichiometric methodology (Lakka et al., 2019a), using DPPH as the radical probe. The ferric-reducing power was measured with a modified FRAP assay and expressed as μmol ascorbic acid equivalents (AAE) per g dry mass (Lakka et al., 2019a).

4.2.9 Chromatographic analyses

The equipment used was a a FinniganMAT P4000 pump, coupled with a UV6000LP diode array detector (Thermo Scientific, Waltham, MA, U.S.A.), and a TSQ Quantum Access LC/MS/MS, with a Surveyor pump (Thermo Scientific, Walltham, MA, U.S.A.), interfaced by XCalibur 2.1, TSQ 2.1 software. Chromatographic analyses were performed on a Superspher RP-18 column, 125 mm × 2 mm, 4 μm, at 40 ºC, with a 10-μL injection loop. The eluents were (A) 2.5% acetic acid and (B) methanol. The flow rate was 0.3 mL min^{-1}, and the elution program used was: 0 min, 100%A; 22 min, 65% A; 32 min, 65% A; 60 min, 0% A; 65 min, 0% A. Mass spectra acquisition was performed with negative ionization, capillary temperature 300 ºC, sheath gas pressure 30 mTorr, auxiliary gas pressure 15 mTorr, and collision pressure at 1.5 mTorr;. Quantification was done with external standards, using a rosmarinic acid (50 – 3000 μg L^{-1}, R^2 = 0.9985)

and a luteolin 7-*O*-glucoside (5 – 1500 μg L^{-1}, R^2 = 0.9982) calibration curve. Standards were prepared in HPLC grade methanol and stored at – 17 ºC.

4.2.10 Statistics

Design of experiment, statistics associated with response surface methodology (ANOVA, lack-of-fit) and distribution analysis was performed with JMP™ Pro 13 (SAS, Cary, NC, USA). Linear regressions, non-linear regressions and kinetics model fitting were performed with SigmaPlot™ 12.5 (Systat Software Inc., San Jose, CA, USA). Extraction experiments were carried out at least twice and all determinations in triplicate. Values given are averages ± standard deviation.

4.3 Results and discussion

4.3.1 Screening of DES for extraction efficiency

The evidence emerged from a previous investigation suggested that citrate salts may form DES with increased extraction efficiency [15]. Thus, the generation of a series of DES was systematically approached, by selecting two widely used HBDs, glycerol (GL) and L-lactic acid (LA), and citrate salts as the HBAs (**Figure 4.1**). The salts tested were sodium citrate monobasic (SCMB), sodium citrate dibasic (SCDB) and sodium citrate tribasic (SCTB). However, as attempts to synthesize DES with either GL or LA and SCMB did not meet with success, even when HBD:HBA molar ratio ($R_{mol}^{D/A}$) was 15, SCMB was not further considered. With regard to SCDB, it formed stable DES (no crystallization) with GL and LA at $R_{mol}^{D/A} \geq 9$; therefore, a series of GL-SCDB and LA-SCDB DES were synthesized with $R_{mol}^{D/A}$ varying from 9 to 15. On the other hand, SCTB formed stable DES with GL only at a $R_{mol}^{D/A}$ of 15. By contrast, stable DES with LA and SCTB were formed at $R_{mol}^{D/A} \geq 7$. Thus, LA-SCTB DES were tested within a $R_{mol}^{D/A}$ range of 7 to 15.

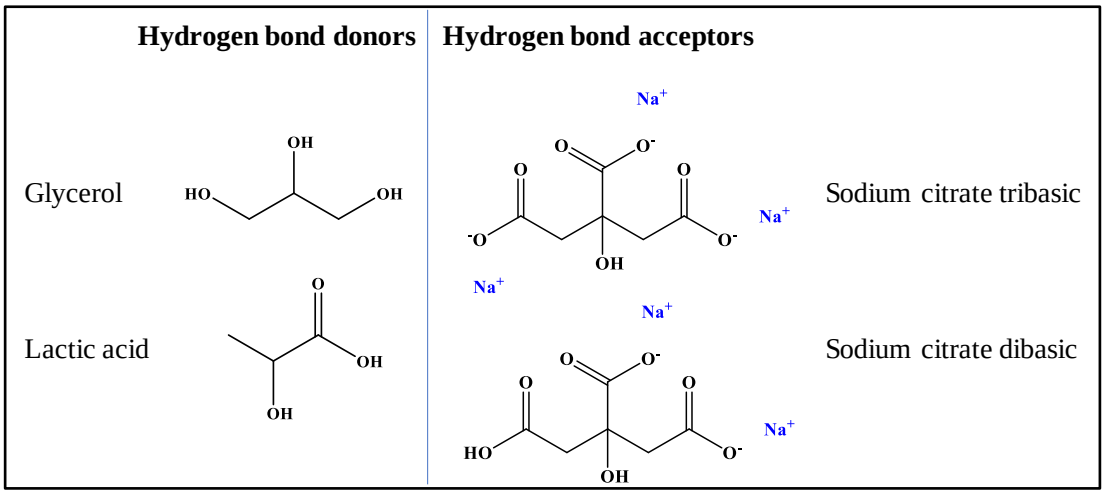


Figure 4.1: Hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) tested in the current investigation.

In total, 14 DES were tested covering a wide pH range, from 2.86 (LA-SCDB15) to 7.50 (GL-SATB15). The extraction efficiency of the DES synthesized was compared to other green solvents, including water and 60% (v/v) ethanol, but also 60% (v/v) methanol, which is a commonly used solvent for polyphenol extraction (**Figure 4.2**).

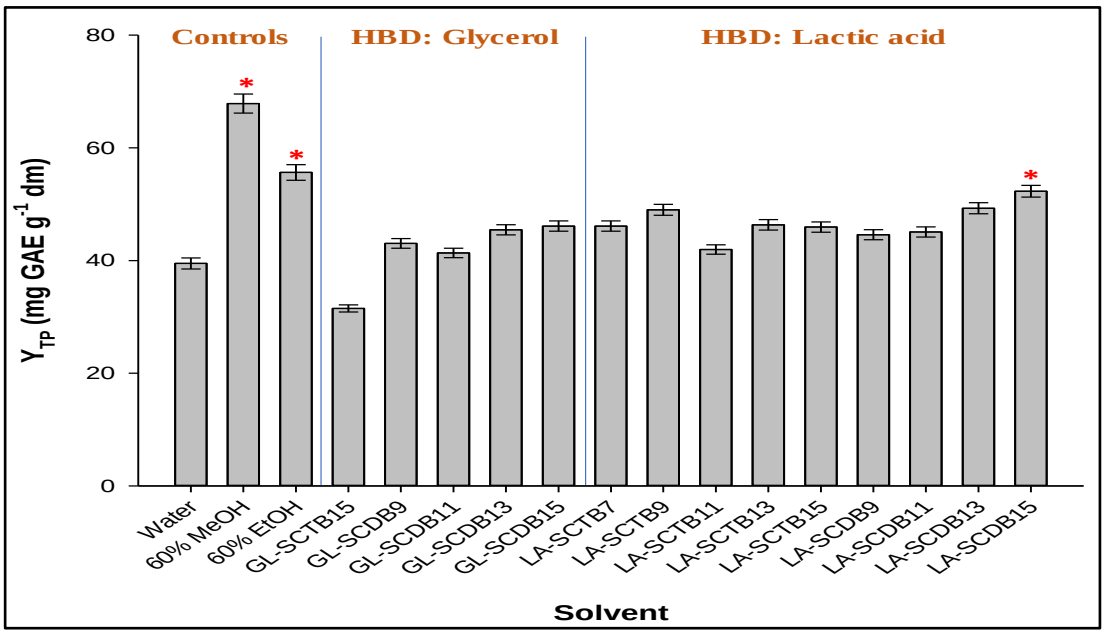


Figure 4.2: Graph showing the results of screening of the DES tested. Extractions were accomplished at 50 ºC, for 150 min, under continuous stirring at 500 rpm. All DES were tested as 70% (w/v) aqueous mixtures. Asterisk (*) signifies statistically different values ($p < 0.05$).

The highest Y_{TP} was found for the extraction with 60% (v/v) methanol (67.86±1.70 mg GAE g^{-1} dm), followed by 60% (v/v) ethanol (55.64±1.39 mg GAE g^{-1} dm). Regarding the DES, the LA-SCDB with $R_{mol}^{D/A}$ = 15, termed as LA-SCDB15, gave a Y_{TP} of 52.31±1.31 mg GAE g^{-1} dm and it was the most efficient one ($p < 0.05$), as opposed to GL-SATB15, which was the least efficient (31.49±0.63 mg GAE g^{-1} dm). Because it was observed that these extreme Y_{TP} values coincided with the corresponding extreme pH values, concerns were raised as to what extent the pH of a DES could affect polyphenol extractability.

To obtain evidence for such an effect, the pH values of all DES tested were plotted against Y_{TP} (**Figure 4.3**). The linear regression gave R^2 = 0.67 (p = 0.0003), revealing a trend that should not be overlooked, which evidenced higher extraction efficiency for DES with lower pH. Although previous studies on polyphenol extraction with DES stressed emphatically the importance of $R_{mol}^{D/A}$ on the extraction yield (Lakka et al., 2019a; Lakka et al., 2019b; Lakka et al., 2020; Kaltsa et al., 2020a; Kaltsa et al., 2020b), a correlation of yield with pH is heretofore unreported.

Earlier investigations with conventional volatile solvents addressed the role of pH on polyphenol extractability, demonstrating that higher total polyphenol yield from olive leaves could be achieved at pH 2

(Mylonaki et al., 2008). Results from following studies on onion solid wastes were in the same line, indicating pH 2 as optimal to maximize polyphenol extraction (Kiassos et al., 2009). Furthermore, examinations on grape stem (Karvela et al., 2009a; Karvela et al., 2009b) and grape seed (Karvela et al., 2009b) polyphenol extraction showed that in most cases higher yields in total polyphenols, total flavanols and proanthocyanidins were favored at pH < 3.5. Such a phenomenon was ascribed to the protective effect of low pH on polyphenol against polyphenol oxidation, because polyphenol oxidizability is higher at neutral or alkaline environment, due to phenolic hydroxyl dissociation. Likewise, it could be argued that acidic DES might act protectively with regard to polyphenol oxidation, and this would be likely to contribute to achieving higher Y_{TP}.

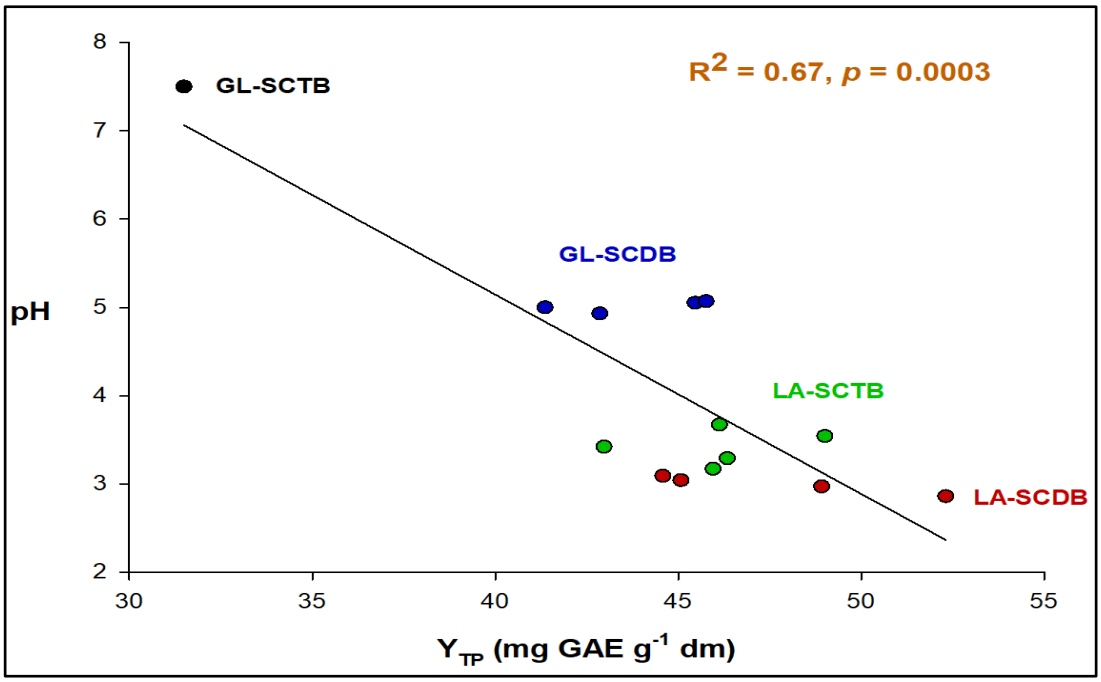
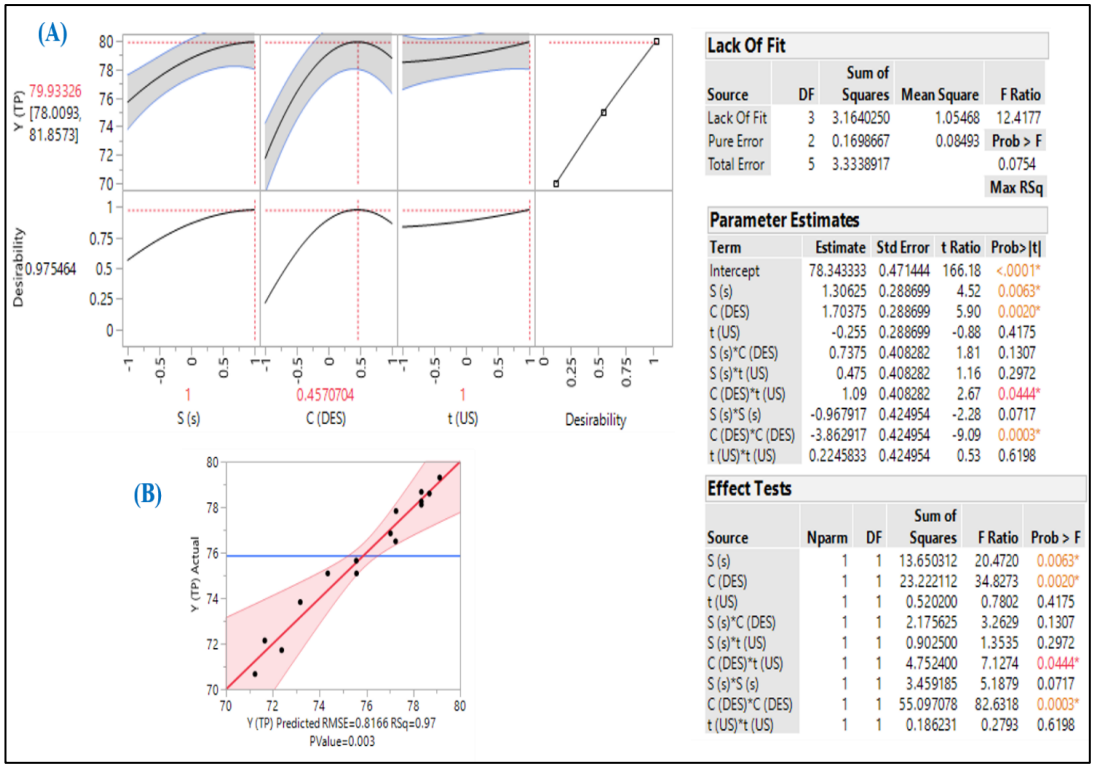


Figure 4.3: Linear regression between the pH of the DES tested and YTP. All DES were tested as 70% (w/v) aqueous mixtures.

4.3.2 Extraction process optimization

Since LA-SCDB15 provided significantly higher Y_{TP} compared to all other DES tested, this solvent was chosen to further optimize the extraction process. To this end, three process variables that can critically affect polyphenol extraction (Lakka et al., 2019a; Kaltsa et al 2020a; Kaltsa et al 2020b), namely the S_S, the C_{DES} and the t_{US}, were included in the experimental design. The design deployed aimed at assessing the effect of the process variables and identifying any synergistic functions between them. The evaluation of model fitting and validity was based on the ANOVA and lack-of-fit test (**Figure 4.4**), taking into account the proximity of measured and predicted values (**Table 4.2**). The mathematical model derived after omitting non-significant terms, was as follows:

$$Y_{TP} = 78.34 + 1.31X_1 + 1.70X_2 + 1.09X_2X_3 - 3.86X_2^2 \ (R^2 = 0.97, p = 0.003) \qquad (4\text{-}6)$$



Lack Of Fit

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	3	3.1640250	1.05468	12.4177
Pure Error	2	0.1698667	0.08493	Prob > F
Total Error	5	3.3338917		0.0754
				Max RSq

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	78.343333	0.471444	166.18	<.0001*
S (s)	1.30625	0.288699	4.52	0.0063*
C (DES)	1.70375	0.288699	5.90	0.0020*
t (US)	-0.255	0.288699	-0.88	0.4175
S (s)*C (DES)	0.7375	0.408282	1.81	0.1307
S (s)*t (US)	0.475	0.408282	1.16	0.2972
C (DES)*t (US)	1.09	0.408282	2.67	0.0444*
S (s)*S (s)	-0.967917	0.424954	-2.28	0.0717
C (DES)*C (DES)	-3.862917	0.424954	-9.09	0.0003*
t (US)*t (US)	0.2245833	0.424954	0.53	0.6198

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
S (s)	1	1	13.650312	20.4720	0.0063*
C (DES)	1	1	23.222112	34.8273	0.0020*
t (US)	1	1	0.520200	0.7802	0.4175
S (s)*C (DES)	1	1	2.175625	3.2629	0.1307
S (s)*t (US)	1	1	0.902500	1.3535	0.2972
C (DES)*t (US)	1	1	4.752400	7.1274	0.0444*
S (s)*S (s)	1	1	3.459185	5.1879	0.0717
C (DES)*C (DES)	1	1	55.097078	82.6318	0.0003*
t (US)*t (US)	1	1	0.186231	0.2793	0.6198

Figure 4.4: Statistics associated with model fitting, performed by implementing response surface methodology. (A), desirability function; (B), actual-by-predicted plot. Inset tables (lack-of-fit, parameter estimates and effect test) illustrate the effect of independent (process) variables on the response. Asterisk (*) on values in the "Parameter estimates" and "Test effects" inset tables signify statistically significant values (at least at a 95% significance level).

The square correlation coefficient (R^2) was a good indicator of the total variability around the mean provided by the equation (4-6). Assuming a confidence interval of 95% and considering the R^2 the *p* value for lack-of-fit (**Figure 4.4**), it could be supported that the mathematical model displayed very satisfactory adjustment to the experimental data. The 3D graphs that represent a visualization of the model, can portray at-a-glance the effect of the process variables on the response (Y_{TP}) (**Figure 4.5**). The desirability function (**Figure 4.4**) provided the theoretical optimized values for each of the variables considered, which were S_S = 900 rpm, C_{DES} = 77% (w/v), and t_{US} = 15 min. By adjusting these optimal settings, the predicted maximum response was calculated to be 79.93±1.92 mg GAE g^{-1} dm. To ascertain the validity of the model, three individual extracts were performed using the optimized values and the outcome was 78.39±2.96 mg GAE g^{-1} dm, illustrating the accuracy of response prediction.

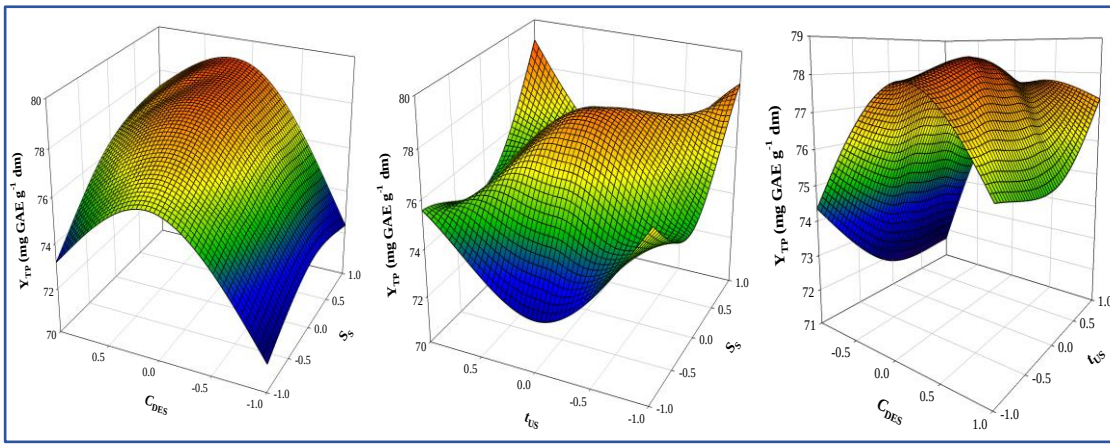


Figure 4.5: The effect of independent variables on the response (YTP), illustrated as three-dimentional plots.

To the contrary, the requirement in S_S for optimum extraction of olive leaf polyphenol was shown to be either low (300 rpm) (Chacroun et al., 2019) or moderate (500 rpm) (Kaltsa et al., 2020a). In another study on the extraction of polyphenols from *M. oleifera* leaves, the optimum S_S was determined to be 800 rpm, but when ultrasonication was integrated as pretreatment, the optimum S_S was 200 rpm (Lakka et al., 2019b). In general, S_S is considered to play important role in solid–liquid extraction, and its careful adjustment may end up in significantly higher yields (Shewale et al., 2018; Vetal et al., 2012).

C_{DES} had also a significant positive effect on Y_{TP}, and the optimum value estimated was 77% (w/v). This level lies between 75 and 80% (w/v) found for polyphenol extraction with DES from *M. oleifera* leaves (Lakka et al., 2020), and 78 and 80% (w/v) from olive leaves (Kaltsa et al., 2020; Athanasiadis et al., 2018)). Other investigations reported 80% (w/w) for tartary buckwheat hull (Y.Huang et al., 2017), 80% (w/w) for sea buckthorn leaves (Cui et al., 2018), 74% (w/w) for *Cymbidium kanran* (K.Jeong et al., 2018) and 76.2% (w/w) for grape skin (K.Jeong et al., 2015). In all these optimization studies, the appropriate adjustment of water amount was shown to be critical for the extraction efficiency, because the DES/water proportion regulates features such as viscosity and polarity (Espino et al., 2016), which profoundly affect solute solubility, hence extraction performance. Such hypothesis has been well exemplified by a recent examination, which demonstrated that the higher the lipophilicity of the HBA in a DES, the higher the water amount required to achieve polyphenol extraction maximization from *O. dictamnus* (Slim et al., 2018).

Table 4.2: Analytical presentation of the design of experiment (design points), including predicted and measured values of the response.

Design point	Independent variables			Response	
	X_1 (S_S, rpm)	X_2 (C_{DES}, % w/v)	X_3 (t_{US}, min)	Y_{TP} (mg GAE g^{-1} dm)	
				Measured	Predicted
1	-1 (300)	-1 (55)	0 (10)	70.67	71.24
2	-1 (300)	1 (85)	0 (10)	73.83	73.17
3	1 (900)	-1 (55)	0 (10)	71.72	72.38
4	1 (900)	1 (85)	0 (10)	77.83	77.26
5	0 (600)	-1 (55)	-1 (5)	75.09	74.35
6	0 (600)	-1 (55)	1 (15)	72.14	71.66
7	0 (600)	1 (85)	-1 (5)	75.09	75.57
8	0 (600)	1 (85)	1 (15)	76.50	77.24
9	-1 (300)	0 (70)	-1 (5)	76.85	77.02
10	1 (900)	0 (70)	-1 (5)	78.60	78.69
11	-1 (300)	0 (70)	1 (15)	75.65	75.56
12	1 (900)	0 (70)	1 (15)	79.30	79.13
13	0 (600)	0 (70)	0 (10)	78.67	78.34
14	0 (600)	0 (70)	0 (10)	78.25	78.34
15	0 (600)	0 (70)	0 (10)	78.11	78.34

It has been supported that a sufficient level of S_S results in turbulence in the extraction tank, which is appropriate to boost mass transfer rate, and increases in S_S have been correlated to higher polyphenol diffusivity (Shewale et al., 2018).

4.3.3 Extraction kinetics – Temperature effects

Previous studies on the extraction of polyphenols from *S. fruticosa* using methyl β-cyclodextrin (m-β-CD) showed that extracts with increased polyphenol concentration and improved antioxidant characteristics could be obtained at 80 ºC (Grigorakis et al., 2020a). However, a following investigation with a 60% (w/v) hydroglycerolic mixture demonstrated that Y_{TP} displayed a gradual decrease when extraction temperature varied from 50 to 80 ºC (Grigorakis et al., 2020b), although differences were non-significant ($p < 0.05$). Therefore, to obtain a reliable picture of the effect of temperature, extraction kinetics was traced within the range of 40 to 80 ºC (**Figure 4.6**), under optimized conditions, that is, S_S = 900 rpm, C_{DES} = 77% (w/v), and t_{US} = 15 min.

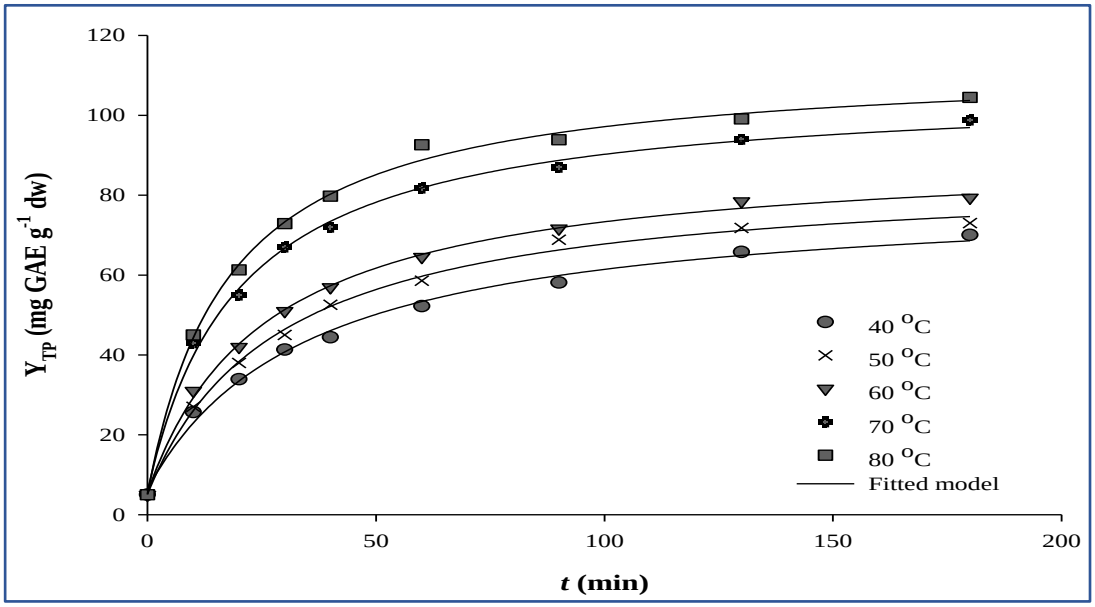


Figure 4.6: Kinetics of polyphenol extraction from S. fruticosa, traced under optimized conditions (S_S = 900 rpm, C_{DES} = 77% (w/v), and t_{US} = 15 min).

Y_{TP} exhibited an increasing trend, and at 80 ºC the $Y_{TP(s)}$ determined was 113.39 mg GAE g^{-1} dm (**Table 4.3**). Likewise, the initial extraction rate, *h*, increased from 2.314 mg g^{-1} min^{-1} at 40 ºC to 6.439 mg g^{-1} min^{-1} at 80 ºC, and $t_{0.5}$ showed a declining tendency over this range, which manifested acceleration of the extraction. In a similar manner, the second-order extraction rate, *k*, increased from 0.356×10^{-3} g mg^{-1} min^{-1} at 40 ºC to 0.501×10^{-3} g mg^{-1} min^{-1} at 80 ºC, and *k* values correlated well with *T* (R^2 = 0.96, p = 0.0413), using the exponential model described by the equation 4-4 (**Figure 4.7**). Comparison

with extraction using hydroglycerolic solvent (Grigorakis et al., 2020b) showed that the fitting parameter b [equation (4)], which is a measure of the sensitivity of *k* with regard to *T* changes, was 0.0136 for the extraction with LA-SCDB15 and 0.0765 for the extraction with hydroglycerolic solvent. This finding suggested that the extraction with hydroglycerolic solvent was more energy-demanding.

To corroborate this hypothesis, the activation energy (E_a) of the process was estimated using the equation x. The barrier level of 7.64 kJ mol^{-1} found was significantly lower than 47.67 kJ mol^{-1} determined for the extraction with 60% (w/v) glycerol (, thus affirming the higher efficiency of the extraction with LA-SCDB15. At this point, it should be stressed that in both cases stirred-tank extraction took place after ultrasonication pretreatment. This pretreatment stage resulted in washing out the most readily extracted compounds, a phenomenon also observed in other cases (Kaltsa et al., 2020; Lakka et al.,2019) and therefore the E_a determined corresponded to the extraction of the remaining solute, whose dissolution and entrainment into the liquid phase is governed by internal diffusion. The fact that the stirred-tank stage was far less energy-demanding using LA-SCDB15 than 60% (w/v) glycerol, evidenced that this solvent might provide higher polyphenol solubility or that it might penetrate easier into the solid particles, or both.

4.3.4 Polyphenolic profile and antioxidant activity – Comparative assessment

To further bring out the efficiency of LA-SCDB15, the characteristics of an extract obtained under optimized conditions were compared to those from two preexisting green extraction methods, one performed with methyl β-cyclodextrin (m-β-CD) [14] and one with 60% (w/v) glycerol/water mixture (GL) (Grigorakis et al., 2020b), but also 60% (v/v) aqueous ethanol and 60% (v/v) aqueous methanol (Σφάλμα! Το αρχείο προέλευσης τ ης αναφοράς δεν βρέθηκε.).

Table 4.3: Illustration of the data derived by implementing kinetics to assess the effect of T on the extraction of S. fruticosa polyphenols, under optimized conditions.

***T* (°C)**	**Kinetic parameters**				
	***k* (× 10^{-3})**	***h***	**$Y_{TP(s)}$**	**$t_{0.5}$**	**E_a**
	(g mg^{-1} min^{-1})	**(mg g^{-1} min^{-1})**	**(mg GAE g^{-1})**	**(min)**	**(kJ mol^{-1})**
40	0.356	2.314	80.64	34.85	
50	0.407	2.994	85.73	28.63	
60	0.424	3.508	90.95	25.93	7.64
70	0.471	5.388	107.01	19.86	
80	0.501	6.439	113.39	17.61	

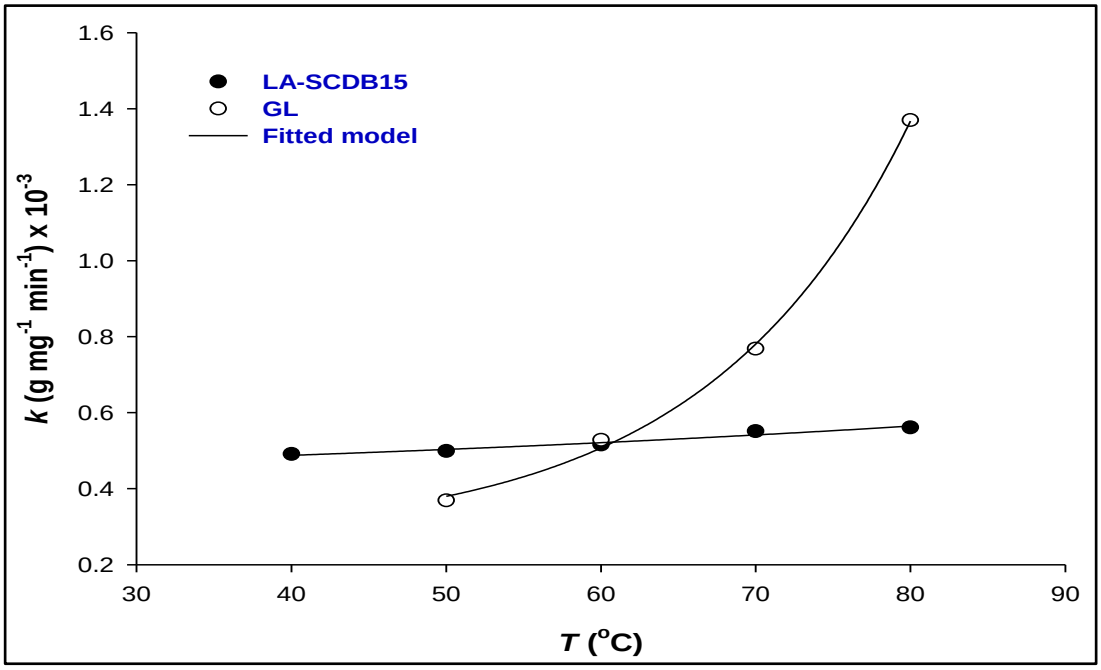


Figure 4.7: Non-linear regression between second-order extraction rate values, k, and T. Data concerning the extraction with 60% glycerol/water (GL) were obtained from Grigorakis et al., 2020.

Table 4.4: Comparative assessment of S. fruticosa extracts produced with LA-SCDB15 and other green solvents. Values given represent means ± standard deviation.

Extract	**Y_{TP}** (mg GAE g^{-1} dm)	**A_{AR}** (µmol DPPH g^{-1} dm)	**P_R** (µmol AAE g^{-1} dm)
Water	63.72±0.96	613.07±12.26[a]	529.14±7.94 [a]
60% MeOH	84.71±1.27	828.54±8.29	703.98±10.56
60% EtOH	87.66±1.31	820.45±16.41	684.20±10.26
m-β-CD	85.54±1.28	820.93±16.42	590.66±14.77
GL	87.26±1.31	817.58±8.18	709.12±17.73
LA-SCDB15	98.05±1.47[a]	751.74±7.52	521.85±7.83[a]

Subscript ([a]) signifies statistically different values ($p < 0.05$) within columns.

The LA-SCDB15 extract was found to have significantly higher Y_{TP}, which demonstrated its high extraction capacity. Furthermore, the extract displayed A_{AR} comparable to the other extracts, except for water extract, whose A_{AR} was significantly weaker. On the other hand, both LA-SCDB15 and water extracts exhibited significantly lower P_R.

Three major *S. fruticosa* polyphenols occurring in LA-SCDB15 extracts were considered for quantification (**Figure 4.8**), and the results were compared to GL and m-β-CD. As can be seen in Σφάλμα! Το αρχείο προέλευσης της αναφοράς δεν βρέθηκε., extraction w ith LA-SCDB15 afforded by 31.8% higher yield in chlorogenic acid compared to m-β-CD, but by 8.3% less so compared to GL. On the other hand, the yield attained with LA-SCDB15 for luteolin 7-*O*-glucuronide was by only 2.7% higher than that attained with m-β-CD, but by 23% higher than that achieved with GL. Likewise, extraction with LA-SCDB15 performed by 38 and 37.6% higher than that with m-β-CD and GL, respectively, with regard to rosmarinic acid recovery. Overall, the extraction with LA-SCDB15 was by 27.6 and 32.9% more efficient than the corresponding carried out with m-β-CD and GL.

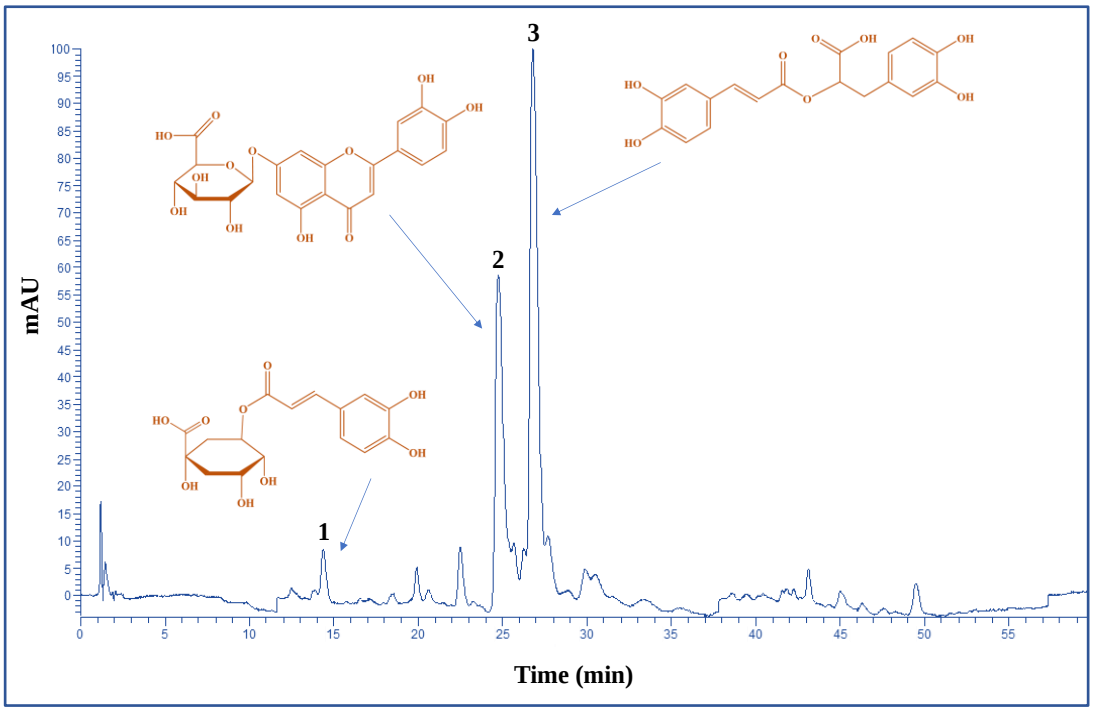


Figure 4.8: Chromatographic analysis of polyphenols in a S. fruticosa extract, produced under optimized conditions (S_S = 900 rpm, C_{DES} = 77% (w/v), and t_{US} = 15 min). The chromatogram was obtained at 330 nm. Peak assignment: 1, chlorogenic acid; 2, luteolin 7-O-glucuronide; 3, rosmarinic acid.

Table 4.5: Extraction yield in principal polyphenolic phytochemicals of S. fruticose, using LA-SCDB15, methyl β-cyclodextrin (m-β-CD) and 60% (w/v) glycerol/water (GL). Values reported are means ± standard deviation.

Compound	**Yield (mg g^{-1} dm) ± sd**		
	m-β-CD	**GL**	**LA-SCDB15**
Chlorogenic acid	0.15±0.02	0.24±0.05	0.22±0.00
Luteolin7-*O*glucuronide	6.96±1.12	5.51±1.57	7.15±0.37
Rosmarinic acid	10.57±1.37	10.63±0.98	17.04±0.15
Sum	17.68	16.38	24.41

This outcome pointed out the higher efficiency of LA-SCDB15 and it was in line with earlier examinations, which demonstrated that polyphenol extraction with DES was more

effective than those performed with common conventional solvents, such as aqueous methanol or ethanol (Lakka et al., 2019a; Kaltsa et al 2020a; Kaltsa et al 2020b, Lakka et al., 2020). At this point it should be stressed that the content of *S. fruticosa* in certain major polyphenolic phytochemicals depends to a large extent by the time of collection. For example, it has been illustrated that the content of rosmarinic acid, which is the main *S. fruticosa* polyphenol, may vary from 5.57 to as high as 45.06 mg g^{-1} dm, and that of chlorogenic acid from 0.46 to 1.82 mg g^{-1} dm (Sarrou et al., 2016). Seasonal ranges between 4.73 and 6.29, and 0.042 and 0.15 mg g^{-1} dm, for rosmarinic and chlorogenic acid, respectively, have also been determined (Dincer et al., 2012). However, other authors reported seasonal variation of rosmarinic acid to be between 0.20 – 1.70 mg g^{-1} dm (Papageorgiou et al., 2008). Levels of rosmarinic acid reported in Greek *S. fruticosa* specimens were 14.83 mg g^{-1} dm (Exarchou et al., 2002) and 27.8 – 76.6 mg g^{-1} dm (Pizzale et al., 2002).

4.4 Conclusions

In the study presented herein, there has been a systematic approach to identify the most effective DES for the extraction of *S. fruticosa* polyphenols, by screening several citrate salts combined with two common HBDs, lactic acid and glycerol. The highest performing system was a DES composed of lactic acid and sodium citrate dibasic, at a molar ratio of 15:1, and for the first time, there has been evidence that the extraction performance of DES might depend on their pH. Optimization of the extraction and examination of the effect of temperature showed that blending ultrasonication pretreatment with optimized stirred-tank extraction may be a highly efficient green method to produce polyphenol-enriched extracts from *S. fruticosa*. This was also demonstrated by comparison with other pre-existing green extraction methodologies. The major polyphenolic phytochemicals identified in the extracts produced under optimized conditions were chlorogenic acid, luteolin 7-*O*-glucuronide and rosmarinic acid. The method developed is proposed as a green and efficacious methodology to recover bioactive polyphenols from the medicinal plant *S. fruticosa*. Testing of this solvent on several other matrices and comparison with other natural DES may reveal its full potential. Such a work is currently under progress.

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CHAPTER 5. Stability of *Salvia fruticosa* Mill. polyphenols and antioxidant activity in a citrate-based natural deep eutectic solvent

Abstract

Previous studies demonstrated that a novel deep eutectic solvent (DES), composed of lactic acid and sodium citrate dibasic at a molar ratio of 15:1 (LA-SCDB15), was a high-performing system with regard to polyphenol extraction from the medicinal plant *Salvia fruticosa* (Greek or Cretan sage). However, an issue of particular importance that should be addressed is the stability of the extract in this novel liquid, since the information available to-date on extract stability in DES is rather limited and inconclusive. This study demonstrated the remarkable stability of the extract, by performing an accelerated and a long-term stability test. In both assays, the antiradical activity and the ferric-reducing power of the extracts were shown to suffer virtually trivial modifications, highlighting the stabilizing effect of LA-SCDB15. Further analytical examination with liquid chromatography-diode array-tandem mass spectrometry assured that the major polyphenolic phytochemicals occurring in *Salvia fruticosa* extracts underwent non-significant changes and remained practically intact. It was concluded that the neoteric DES LA-SCDB15 may provide outstanding stability in polyphenol-containing extracts and its testing on other plant extracts is proposed as a further step towards revealing its stabilizing potential.

Keywords: antioxidant activity; deep eutectic solvents; polyphenols; *Salvia fruticosa*; stability

5.1 Introduction

Polyphenols are inherently molecules prone to oxidation and/or other structural modifications, and thus the study of polyphenol stability under a given set of conditions is of paramount importance. Recently, our group reported the use of a novel DES, coded LA-SCDB15, which displayed very high performance in the extraction of polyphenols from *S. fruticosa* (Grigorakis *et al*. 2020). To further appraise the applicability of this solvent in the generation of polyphenol-enriched extracts, this study was undertaken to examine the stability of *S. fruticosa* polyphenols in extracts produced using LA-SCDB15. Stability was assessed by monitoring the antiradical activity and the ferric-reducing power of the extracts, by employing an accelerated and a long-term test, at various temperatures. Moreover, to better illustrate the effect of extract storage in LA-SCDB15, liquid chromatography-diode array-tandem mass spectrometry (LC-DAD-MS/MS) analyses were also performed, to trace changes in major polyphenolic phytochemicals.

5.2 Experimental

5.2.1 Chemicals

All chromatographic analyses were accomplished with solvents of HPLC grade. L-lactic acid (80%) was from Fisher Scientific (Loughborough, UK). Sodium citrate dibasic sesquihydrate (>99%), rosmarinic acid, sodium carbonate, ascorbic acid, sodium acetate trihydrate, luteolin 7-*O*-glucoside, 2,2-diphenylpicrylhydrazyl (DPPH) and chlorogenic acid were from Sigma-Aldrich (Darmstadt, Germany). Folin-Ciacalteu reagent was from Merck (Darmstadt, Germany). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) and iron chloride hexahydrate were from Honeywell/Fluka (Steinheim, Germany). The deep eutectic solvent (DES), composed of lactic acid (LA) and sodium citrate dibasic (SCDB) at a molar ratio of 15:1, was synthesized as described earlier (Grigorakis *et al*. 2020).

5.2.2 Plant material

Certified *S. fruticosa* was provided by a botanicals store (Chania, Greece). The specimen composed of the aerial parts of the plant and it was received in dried form, in hermetically closed plastic packaging. Upon receipt, the plant material was pulverized in a table mill (Tristar, Tilburg, The Netherlands), as described previously (Grigorakis *et al*. 2020) and stored under refrigeration (4 ºC).

5.2.3 Preparation of polyphenol extracts

Extraction of *S. fruticosa* was performed using the optimized process, as reported elsewhere (Grigorakis *et al*. 2020c). In short, 0.375 g of dried plant material was mixed with 15 mL 77% (w/w) DES/water and ultrasonicated for 15 min in an ultrasonication bath (Sonorex Bandeline, Berlin, Germany), at room temperature (23±2 ºC). The ultrasonication settings were: power, 120 W; frequency, 100 Hz; acoustic energy density, 120 W L^{-1}. After ultrasonication, which was the pretreatment stage, batch stirred tank extraction was carried out at a stirring speed (S_S) of 900 rpm, at 80 ºC, for 150 min. The extract thus obtained was centrifuged for 10 min at 10,000×*g* and the transparent supernatant was used for stability tests.

5.2.4 Determination of the antiradical activity

The assay was performed with a stoichiometric methodology, using DPPH (Cevalos-Casals and Cisneros-Zevallos, 2003; Athanasiadis *et al*. 2017). The total stoichiometries (n_t) of the reaction between DPPH and extract polyphenols were determined using the following equation:

$$n_t = \frac{A_0 - A_f}{\varepsilon \, C_{TP}} \qquad (5\text{-}1)$$

Where C_{TP} is the total polyphenol concentration of the extracts (mg L^{-1}), ε (DPPH) = 11,126 × 10^6 μM^{-1} cm^{-1}, A_0 the A_{515} at t = 0 and A_f the A_{515} at t = 30 min. Results were expressed as μmol DPPH per mg total polyphenols.

5.2.5 Determination of the ferric-reducing power

A previously published protocol was used (Karakashov *et al*. 2015). Briefly, volume of 0.05 mL of ferric chloride (4 mM in 0.05 M HCl) was combined with an equal volume of sample and the mixture was heated up at 37 ºC, in a water bath, for 30 min. After incubation, 0.9 mL of TPTZ solution (1 mM in 0.05 M HCl) was added and the mixture was allowed to stand at room temperature for another 10 min. The absorbance at 620 nm was recorded using suitable control and results were reported as mM ascorbic acid equivalents (AAE).

5.2.6 Total polyphenol concentration

The methodology described elsewhere was implemented (Karakashov *et al*. 2015). In an Eppendorf tube of 1.5 mL, volume of 0.02 mL sample, 0.78 mL deionized water and 0.05 mL Folin-Ciocalteu reagent were mixed and left to react for 2 min, in the dark, at ambient

temperature. Then, 0.15 mL of 20% sodium carbonate was added and the mixture was allowed to stand for 60 min. A calibration curve constructed with gallic acid was used for quantification.

5.2.7 Chromatography

A method previously reported was used (Grigorakis *et al*. 2020a). A FinniganMAT P4000 pump coupled to a UV6000LP diode array detector (Thermo Scientific, Waltham, MA, U.S.A.), and a TSQ Quantum Access LC/MS/MS, equipped with a Surveyor pump (Thermo Scientific, Wallthan, MA, U.S.A.), and controlled by XCalibur 2.1, TSQ 2.1 software, were employed. The column was Superspher RP-18, 125 mm × 2 mm, 4 μm, kept at 40 ºC. The injection volume was 10 μL and the eluents used were (A) 2.5% acetic acid and (B) methanol, operated at a flow rate of 0.3 mL min^{-1}. The elution was as follows: 0 min, 100% A; 22 min, 65% A; 32 min, 65% A; 60 min, 0% A; 65 min, 0% A. Acquisition of mass spectra was done with negative ionization, employing sheath gas pressure 30 mTorr, auxiliary gas pressure 15 mTorr, collision pressure at 1.5 mTorr and capillary temperature 300 ºC. Quantification was performed using a calibration curve of luteolin 7-*O*-glucoside (5 – 1500 μg L^{-1}, R^2 = 0.9982), chlorogenic acid (50 – 1500 μg L^{-1}, R^2 = 0.9986) and rosmarinic acid (50 – 3000 μg L^{-1}, R^2 = 0.9985). All standards were prepared in HPLC grade methanol.

5.2.5 Accelerated stability test

A volume of extract was placed in a glass vial and heated up at 50, 60, 70, 80 and 90 ºC for 240 min, by means of a heating magnetic stirrer (VELP Scientifica, NY, USA). Sampling was accomplished at 30-min intervals to assay antioxidant activity.

5.2.6 Long-term stability test

Equal volumes of extract were transferred into glass vials and stored in the fridge (7 ºC), on the bench (23±2 ºC) and in a water bath adjusted at 40 ºC, for 30 days. During this period, special care was taken to avoid extract contact with light. Sampling was carried out at 3-days intervals to determine antioxidant activity.

5.2.7 Statistical analysis

Procedures were repeated twice, and determinations were carried out in triplicate. Values were given as averages ± standard deviation (sd). Distribution analysis was carried out with JMP™ Pro 13 (SAS, Cary, NC, USA). Linear correlations were accomplished with SigmaPlot™ 12.5 (Systat Software Inc., San Jose, CA, USA).

5.3 Results and discussion

5.3.1 Accelerated stability test

The objective of the test was to ascertain whether the antioxidant properties of the extract could be impacted as a result to exposure to a range of temperatures, varying from moderate (50 ºC) to severe (90 ºC) heating, for 240 min, and thus to draw conclusions regarding extract stability in LA-SCDB15. The results of the test are depicted in **Figure 5.1**. Over the range 50 to 80 ºC, the extract displayed by almost 17.5 - 21% higher A_{AR} compared to the initial (untreated) sample. However, A_{AR} declined to a level equal to the initial one, after heating at 90 ºC. Differences amongst values were shown to be non-significant ($p > 0.05$), which indicated that increases in temperature did not affect A_{AR} to a significant extent. Furthermore, no consistent trend was observed in A_{AR} as a response to temperature. Likewise, P_R remained virtually intact since the differences found amongst the initial extract and the extracts treated at 50 to 90 ºC varied between 0 and 3.1%. This finding strongly suggested that LA-SCDB15 used to produce the extract, provided exceptional stability.

5.3.2 Long-term stability test

This test was employed to trace fluctuations in both A_{AR} and P_R during storage of the extract for 30 days, at different storage temperatures. As can be seen in **Figure 5.2**, the A_{AR} of the extract stored at 7 ºC exhibited a significant decline by 39.9%, from 9.41 (day 0) to 5.66 μmol DPPH mg^{-1} TP (day 12). However, A_{AR} recovered to 8.91 μmol DPPH mg^{-1} TP by the end of the examination period (day 30). On the other hand, the extract stored at ambient conditions (23 ºC) displayed less intense variations and, after an increase to 9.87 μmol DPPH mg^{-1} TP (3.1%) at day 12, it dropped to 8.10 μmol DPPH mg^{-1} TP at day 30. The extract stored at 40 ºC manifested a different pattern, as its A_{AR} increased up to 10.16 μmol DPPH mg^{-1} TP at day 15, but it declined to 8.93 μmol DPPH mg^{-1} TP at day 30.

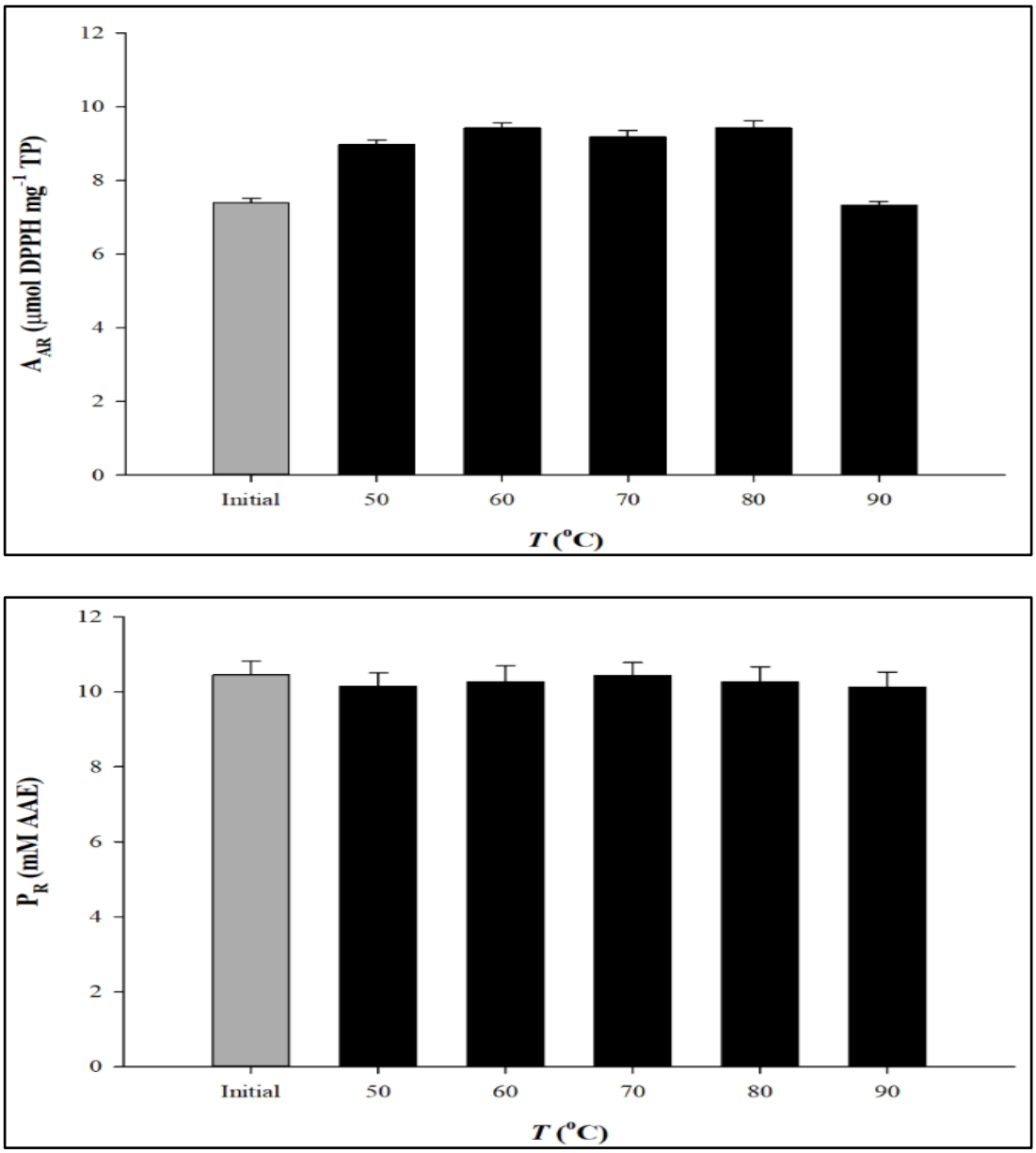

Figure 5.1: Comparison of AAR (upper plot) and PR (lower plot) of S. fruticosa extracts, undergone no treatment (initial) and after treated at 50 – 90 ºC, for 240 min.

Thus, at the end of the treatment the extracts had practically equal A_{AR}, irrespective of the storage temperature. The monitoring of P_R revealed a diversified time course than that seen with A_{AR}. The pattern observed for the samples stored at 7 and 40 ºC was almost identical, and at the end of the treatment, the extracts had P_R of 11.97 and 11.07 mM AAE, respectively. On the contrary, the extract stored at 23 ºC showed rather large variations between day 18 and day 30, while its final level (day 30) was 12.04 mM AAE.

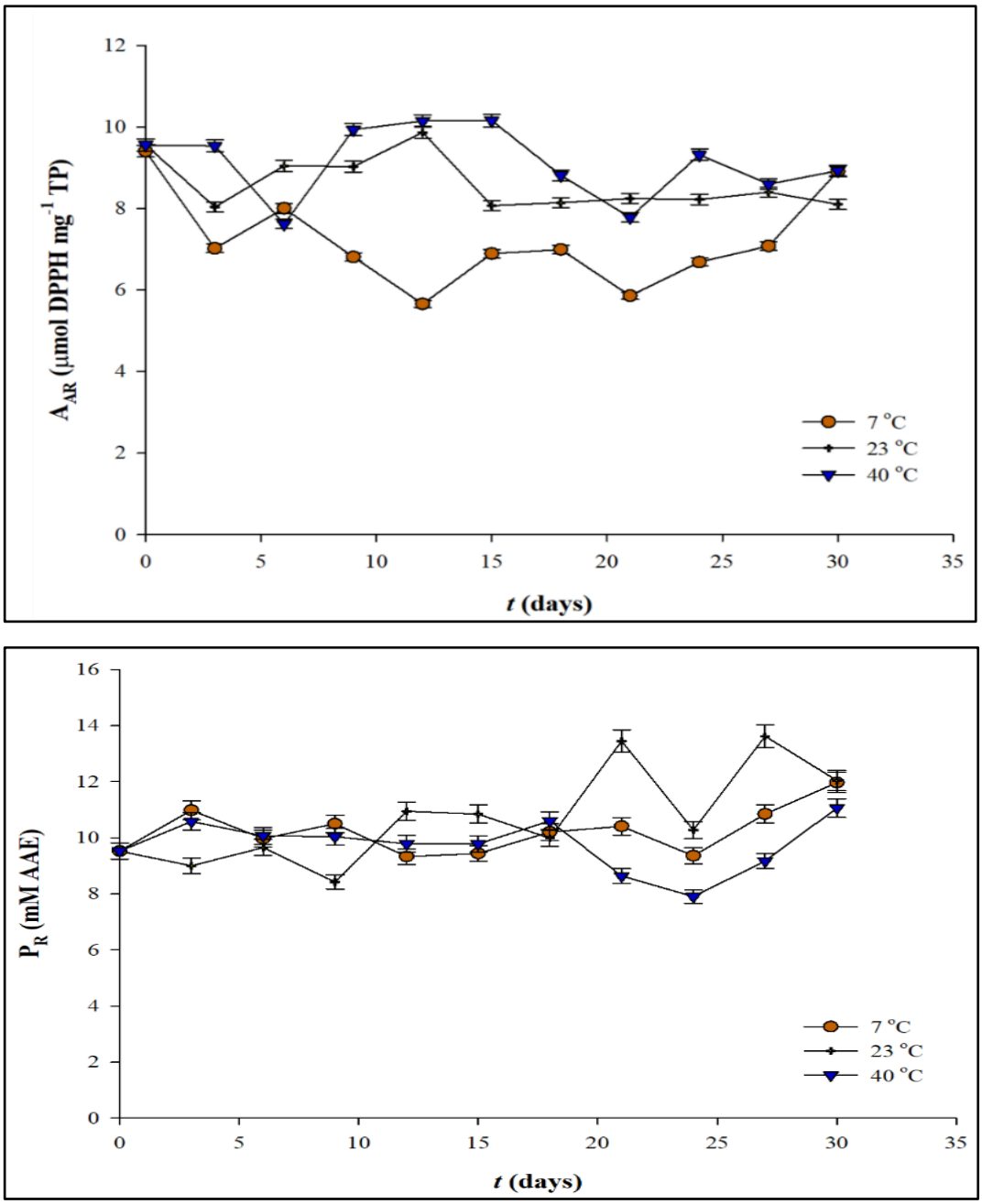

Figure 5.2: Monitoring of AAR (upper plot) and PR (lower plot) of S. fruticosa extracts, stored at 7, 23 and 40 ºC, for 30 days.

The difference amongst P_R values at day 30 and the initial extract (9.53 mM AAE) were low and statistically non-significant ($p > 0.05$), which further confirmed the outstanding stability of the extract in LA-SCDB15.

Early studies evidenced that the antioxidant activity, as evaluated by A_{AR} and P_R, might reflect changes associated with polyphenolic composition, such as oxidation (Sioumis *et al*. 2005). On the other hand, examinations on stability of polyphenol-containing extracts

in DES are particularly limited, but the evidence emerged suggested that DES may provide improved stability over conventional solvents. Such an effect has been demonstrated for safflower (*Carthamus tinctorius*) pigments in a glucose-choline chloride DES (Dai *et al*. 2014) and *Catharanthus roseus* anthocyanins in a lactic acid-glucose DES (Dai *et al.* 2016). Long-term stability studies on *Moringa oleifera* extracts in a glycerol-sodium acetate DES showed that A_{AR} displayed a constant decline over a 18-days storage period, at every temperature tested (4, 22 and 50 ºC), which obeyed pseudo-first order kinetics (Karageorgou *et al*. 2018). At 50 ºC, where the highest declining rate was found, polyphenols were extensively degraded, and the authors argued that this was the reason for the low A_{AR} levels recorded at the end of the treatment. In that case, addition of hydroxypropyl β-cyclodextrin was found to slow down A_{AR} drop. Similarly, olive leaf (*Olea europaea*) extracts in a glycerol-glycine DES showed a decreasing trend in P_R, which followed pseudo-zero order kinetics over a period of 20 days (Athanasiadis *et al*. 2018), irrespective of the assay temperature (4, 22 and 50 ºC). The presence of methyl β-cyclodextrin delayed the progression of the phenomenon, yet at 50 ºC some principal metabolites suffered extensive degradation and a novel yellow pigment was formed.

However, unlike investigations revealing a reduction in either A_{AR} or P_R, other studies illustrated that there was no specific pattern regarding the evolution of antioxidant activity during storage. Monitoring of both A_{AR} and P_R of onion solid waste extracts produced with a glycerol-sodium propionate DES, for a period of 30 days at 22 ºC, showed that at the end of storage A_{AR} was enhanced by 19%, whereas P_R was virtually unaffected (Stefou *et al*. 2019). At the same time, no major changes were observed in the polyphenolic profile of the extracts. On the other hand, olive leaf extracts in a lactic acid-ammonium acetate DES, containing β-cyclodextrin, displayed a striking increase in A_{AR} by 100% after 30 days at 22 ºC, although in this case too, P_R was stable and fluctuated within narrow limits (Chakroun *et al*. 2020). Paradoxically, the major polyphenols in the extract showed a decrease by 4.4 – 42%. On the basis of the above-mentioned, it could be argued that both A_{AR} and P_R exhibited very high stability in LA-SCDB15, which has not been previously encountered.

5.3.3 Polyphenolic composition

To shed more light onto the effect of LA-SCDB15 on the stability of *S. fruticosa* extracts, liquid chromatography-diode array-tandem mass spectrometry (LC-DAD-MS/MS)

analyses were undertaken. The scope of this examination was the detection of alterations in the polyphenolic profile of the extracts stored at different temperatures, as well as quantitative changes in major polyphenolic phytochemicals. Chlorogenic acid and rosmarinic acid were identified by comparing the retention time and UV-vis spectra with those of authentic standards. Their identity was also confirmed by their respective pseudo-molecular ions at *m*/*z* = 353 and 359. Luteolin 7-*O*-glucuronide was tentatively identified considering the pseudo-molecular ion at *m*/*z* = 461 and the aglycone (luteolin) at *m*/*z* = 285 (Grigorakis *et al*. 2020a). The traces, recorded at 330 nm, of the extracts stored at various temperatures (7, 23, 40 ºC), had identical polyphenolic profile and no major differences were seen (**Figure 5.3**)

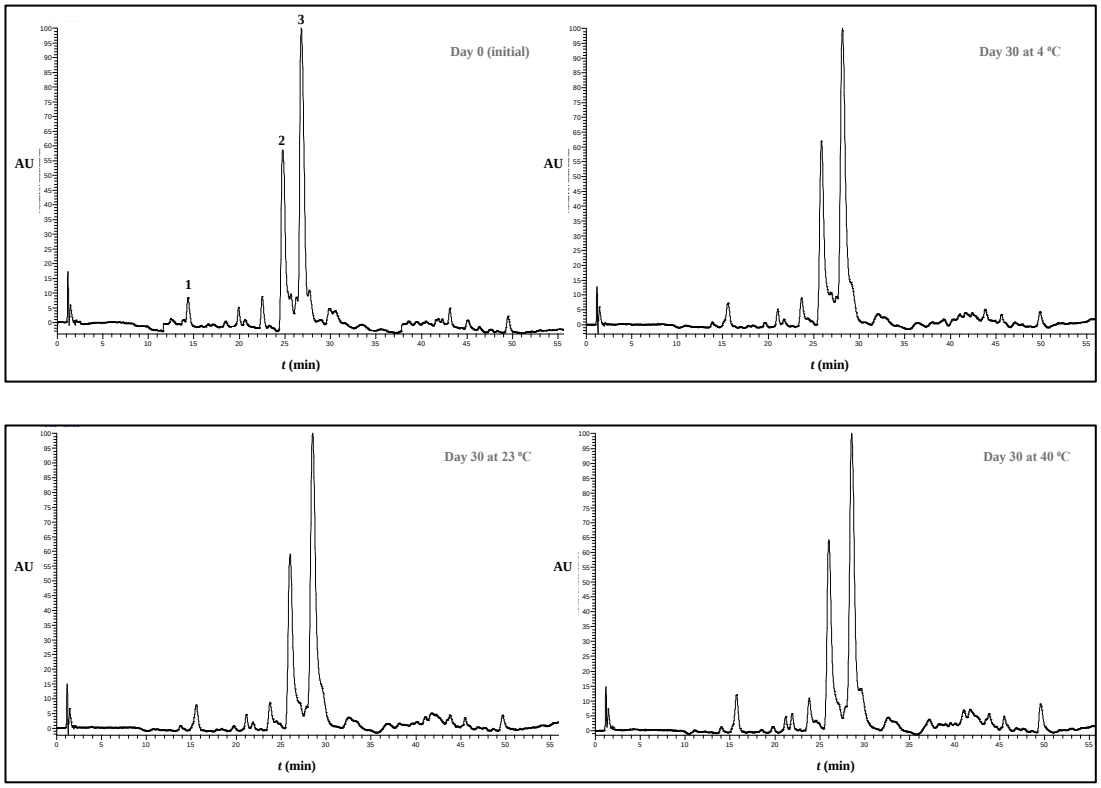

Figure 5.3: Chromatograms of *S. fruticosa* extracts, obtained at 330 nm, illustrating the effect of extract storage at different temperatures after 30 days, on the major polyphenolic compounds. Peak assignment: 1, chlorogenic acid; 2, luteolin 7-*O*-glucuronide; 3, rosmarinic acid.

This outcome indicated neither the extensive decomposition of any of the principal metabolites, nor the formation of any other substance, and evidenced the stability of the extract, irrespective of the storage temperature. To better portray possible changes in chlorogenic acid, luteolin 7-*O*-glucuronide and rosmarinic acid, brough about during storage, a quantitative investigation was also performed (**Table 5.1**). Storage at 7 ºC

resulted in a by 5.6% decrease in the sum of compounds, while at 23 and 40 ºC the corresponding changes were 10.4 and 6.1%. For all compounds considered, the modifications in their concentration found were limited and statistically non-significant, highlighting once again the extraordinary stability of the extract in LA-SCDB15.

Table 5.1: Quantitative data on changes occurred on major *S. fruticosa* polyphenols, after storage of extracts at different temperatures, after 30 days. Values given represent means ± standard deviation.

Compound	**Storage temperature**			
	Initial	**4 ºC**	**23 ºC**	**40 ºC**
Chlorogenic acid	0.085±0.002	0.083±0.006	0.085±0.005	0.082±0.006
Luteolin 7-*O* glucuronide	5.99±0.17	5.47±0.167	5.25±0.31	5.92±0.04
Rosmarinic acid	14.67±0.33	14.05±0.27	13.25±0.33	13.48±0.08
Sum	20.75	19.60	18.59	19.48

5.4 Conclusions

In the current examination, the stability of polyphenol-containing extract from the medicinal plant *S. fruticosa* in a novel DES termed as LA-SCDB15, was studied by deploying an accelerated and a long-term stability test. The accelerated test, performed over a range of temperatures varying from 50 to 90 ºC, provided substantial evidence for exceptional extract stability, since the antioxidant activity remained virtually unaffected upon treatment for 240 min. The long-term test monitored antioxidant activity variations over a period of 30 days at various temperatures and assured that the extract suffered no major alteration in its antioxidant properties. A clear confirmation of stability emerged from LC-DAD-MS/MS analyses, which showed that the major polyphenols occurring in *S. fruticosa* extracts remained practically intact, irrespective of the storage temperature. The study presented herein demonstrated a remarkable stability of polyphenol extracts in the LA-SCDB15. The use of this neoteric solvent regarding effective extract storage remains to be elucidated for other plant materials too. This will confirm the polyphenol-stabilizing ability of this liquid and enable its wider applicability.

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CHAPTER 6. General conclusions

The addition of cyclodextrins demonstrated enhanced extraction capacity for aquatic solutions. The m-β-CD was proven the most efficient extraction booster, providing extracts with significant polyphenol yield and improved antioxidant characteristics, fact that designates the importance of the type of cyclodextrin in the extraction process. Kinetics showed that with (i) extraction performance and antioxidant activity may be even more enhanced at 80 ºC and (ii) extraction with m-β-CD was the least energy demanding. LC/MS/MS analyses revealed that luteolin 7-*O*-glucuronide and rosmarinic acid were the predominant polyphenols in the extracts obtained with either CD, and that m-β-CD might exhibit higher affinity for luteolin 7-*O*-glucuronide, and β-CD for rosmarinic acid. The conclusions drawn may be of value in developing green extraction processes for effective polyphenol recovery, not only for *S. fruticosa*, but also other botanical species possessing similar polyphenolic composition. Furthermore, the selectivity issue concerning various CDs should be more thoroughly tested on plant matrices with variable polyphenolic composition, to study the effect of structural features on polyphenol extractability.

Glycerol is a cheap and safe material that recently gained more attention as an alternative extraction media. Sonication pretreatment increased the extraction yield of salvia polyphenols and may offer a solution to diffusivity and mass transfer problems of high viscosity solvents. The kinetic studies showed that extraction at 50 ºC may be the most favorable, and thus this methodology may also be energy-effective, a fact that significantly adds to the sustainable profile of the process. A prospect of this investigation would be future studies focusing on scale-up and application of hydroglycerolic extracts of *S. fruticosa* as effective food antioxidants/antimicrobials and/or cosmetic constituents.

Deep eutectic solvents exhibit a number of properties, among them, safety and tunability are these that made them appealing as extraction solvents. Novel DES formulations have been synthesized combining glycerol or lactic acid with citrate salts. The highest performing system was a DES composed of lactic acid and sodium citrate dibasic, at a molar ratio of 15:1, and for the first time, there has been evidence that the extraction performance of DES might depend on their pH. Optimization of the extraction and examination of the effect of temperature showed that blending ultrasonication pretreatment with optimized stirred-tank extraction may be a highly efficient green

method to produce polyphenol-enriched extracts from *S. fruticosa*. This was also demonstrated by comparison with other pre-existing green extraction methodologies. The method developed is proposed as a green and efficacious methodology to recover bioactive polyphenols from the medicinal plant *S. fruticosa*.

The stability of polyphenol-containing extract from the medicinal plant S. fruticosa in a novel DES termed as LA-SCDB15, was studied by deploying an accelerated and a long-term stability test. The accelerated test, performed over a range of temperatures varying from 50 to 90 ºC, provided substantial evidence for exceptional extract stability, since the antioxidant activity remained virtually unaffected upon treatment for 240 min. The long-term test monitored antioxidant activity variations over a period of 30 days at various temperatures and assured that the extract suffered no major alteration in its antioxidant properties. A clear confirmation of stability emerged from LC-DAD-MS/MS analyses, which showed that the major polyphenols occurring in S. fruticosa extracts remained practically intact, irrespective of the storage temperature.

Appendix

i) Published work in international scientific journals with referees

1. High-Performance Green Extraction of Polyphenolic Antioxidants from *Salvia fruticosa* Using Cyclodextrins: Optimization, Kinetics,and Composition.

Spyros Grigorakis, Amina Benchennouf , Abedalghani Halahlah and Dimitris P. Makris

Journal name: *Applied Sciences*

Publication date: 16 May 2020

DOI: https://doi.org/10.3390/app10144774

URL: https://www.mdpi.com/2076-3417/10/14/4774

2. Hydroglycerolic Solvent and Ultrasonication Pretreatment: A Green Blend for High-Efficiency Extraction of *Salvia fruticosa* Polyphenols

Spyros Grigorakis, Abedalghani Halahlah and Dimitris P. Makris

Journal name: *Sustainability*

Publication date: 13 June 2020

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URL: https://www.mdpi.com/2071-1050/12/12/4840

3. Batch Stirred-Tank Green Extraction of *Salvia fruticosa* Mill. Polyphenols Using Newly Designed Citrate-Based Deep Eutectic Solvents and Ultrasonication Pretreatment

Spyros Grigorakis, Abedalghani Halahlah and Dimitris P. Makris

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4. Stability of *Salvia fruticosa* Mill. polyphenols and antioxidant activity in a citrate based natural deep eutectic solvent

Spyros Grigorakis, Abedalghani Halahlah and Dimitris P. Makris

Journal name: Nova Biotechnologica et Chimica

Publication date: In Press

applied sciences

MDPI

Article

High-Performance Green Extraction of Polyphenolic Antioxidants from *Salvia fruticosa* Using Cyclodextrins: Optimization, Kinetics, and Composition

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Abstract: *S. fruticosa*, collectively known as Cretan sage, is a medicinal plant to which a number of bioactivities have been attributed. In spite of its importance in nutrition and pharmacy, reports on the extraction of major polyphenols using sustainable processes are particularly limited. In this study, three common cyclodextrins, namely, methyl β-cyclodextrin (m-β-CD), hydroxypropyl β-cyclodextrin (HP-β-CD), and β-cyclodextrin (β-CD), were tested as green boosters of aqueous extraction of polyphenols from aerial parts of *S. fruticosa*. To examine simultaneously important extraction parameters, including the concentration of cyclodextrins (C_{CD}), pH, and liquid-to-solid ratio ($R_{L/S}$), a Box–Behnken design was chosen, with three central points. Temperature effects on the extraction yield were also considered, by carrying out kinetics. The results showed that m-β-CD was the most effective extraction booster, providing total polyphenols yields that amounted to 98.39 mg gallic acid equivalents g^{-1} dry mass. The kinetic assay demonstrated that extraction was highly effective at 80 °C, increasing significantly polyphenol yield, as well as the ferric-reducing power and antiradical activity of the extracts. It was also proven that extraction with m-β-CD was the least energy-demanding process. Liquid chromatography-tandem mass spectrometry examination revealed that m-β-CD might possess higher affinity for luteolin 7-*O*-glucuronide extraction, but β-CD for rosmarinic acid extraction.

Keywords: antioxidants; cyclodextrins; extraction kinetics; green extraction; polyphenols; *Salvia fruticosa*

1. Introduction

Salvia is the most multitudinous genus of the Lamiaceae family, embracing over than 800 species around the globe [1]. Several tens of *Salvia* species are considered plants with high pharmacological potency, being an integral part of folk medicine in many countries [2]. The therapeutic properties of *Salvia* plants have been mostly attributed to major constituents, such as terpenoids and phenolic acids, yet a wide diversity of flavonoid compounds may also occur in *Salvia* specimens [3,4].

The biological significance of medicinal plants has triggered the development of a high number of extraction techniques, which aim at the effective recovery of polyphenolic substances. These techniques

may involve the use of volatile and toxic solvents, while the extracts obtained may afterwards require several steps of downstream processes for effective solvent removal and extract recovery. On the other hand, contemporary trends in polyphenol extraction, driven by the need for less environmentally aggravating and safer processes, dictate the development of extraction methodologies that would minimize cost, energy consumption, and emission of volatile substances [5]. On this philosophy, the replacement of conventional extraction media by novel, green, and non-toxic ones is imminent.

Cyclodextrins (CDs) are cyclic oligosaccharides, composed of α (1→4)-linked subunits of D-glucopyranose. The most common CDs are α-, β-, and γ-CDs, composed of six, seven, and eight glucose units, respectively, obtained by enzymic degradation of starch, possessing a truncated cone shape, with the hydroxyl functions located towards to the outer cavity surface (Figure 1). This three-dimensional structure of the CD molecules is characterized by a hydrophilic outer surface and an internal hydrophobic cavity and provides both water solubility and ability to encapsulate within the cavity hydrophobic molecules of suitable size, thus forming inclusion complexes [6].

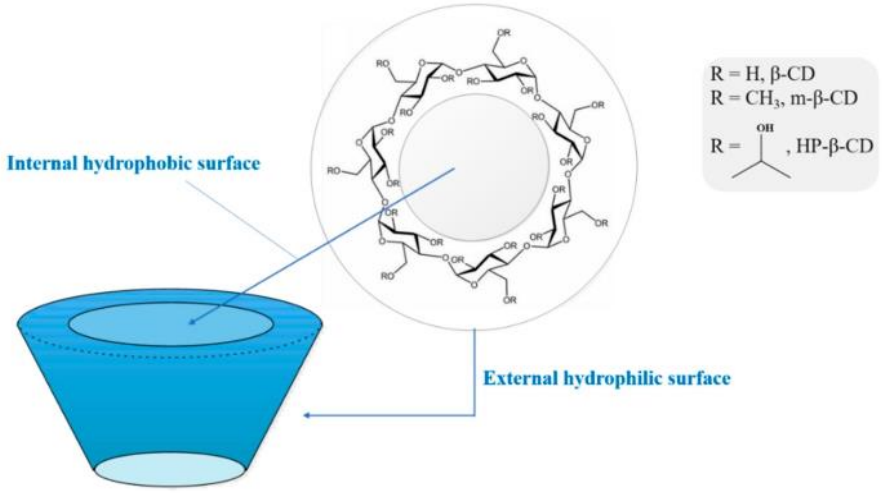

Figure 1. Cyclodextrin structures tested in this study. Assignments: m-β-CD, methyl β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin; β-CD, β-cyclodextrin.

Applications of CDs have been increasing on an annual basis in pharmaceutical, chemical, and other disciplines, but most uses are related to food [7]. The utilization of CDs in food products pertains mainly to stabilization of flavors, solubilization of poorly water-soluble substances, protection of labile additives, and so on. However, CDs use for extraction of polyphenolic compounds is a state-of-the-art trend, offering unprecedented opportunities in the so-called "green extraction". This is because, although common organic solvents regularly used for polyphenol recovery (e.g., ethanol, ethyl acetate) display excellent potency for polyphenols dissolution and extraction, their use poses serious environmental concerns. Aqueous systems containing CDs may be regarded as green solvents, with a prospect of replacing organic solvents in relevant processes [8].

In this frame, this investigation was performed with the objective to study the use of various cyclodextrins, namely, β-cyclodextrin (β-CD), hydroxypropyl β-cyclodextrin (HP-β-CD), and methyl β-cyclodextrin (m-β-CD), on the extraction of polyphenols from *S. fruticosa*, using aqueous solutions. The investigation was based on experimental design considering critical extraction parameters, such as the CD concentration, the liquid-to-solid ratio, and pH. Finally, temperature effects were assayed by carrying out kinetics, while selectivity issues were checked with liquid chromatography-mass spectrometry analyses.

2. Materials and Methods

2.1. Chemicals

β-Cyclodextrin, hydroxypropyl β-cyclodextrin, and methyl β-cyclodextrin were from Sigma–Aldrich (St. Louis, MO, USA). Ethanol (99.8%) was from Acros Organics (Geel, Belgium). Anhydrous sodium carbonate was from Carlo Erba Reactifs (Val de Reuil, France). Folin–Ciocalteu reagent, 2,4,6-tripyridyl-*s*-triazine (TPTZ, 99%) and ferric chloride hexahydrate were from Fluka (Steinheim, Germany). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), citric acid, gallic acid, and ascorbic acid were from Aldrich (Steinheim, Germany). Chromatography solvents were high-performance liquid chromatography (HPLC) grade.

2.2. Plant Material

Cretan sage (*Salvia fruticosa*, Lamiaceae) was provided by a local store (Chania, Crete, Greece) of certified botanicals. The plant material was delivered dried in air-tight packaging and stored in a dark and dry chamber for no longer than a week. The material was pulverised using a table domestic mill (Tristar, Tilburg, The Netherlands) to provide a powder with mean particle size of 1.28 mm. This powder was used for all examinations.

2.3. Batch Stirred-Tank Solid–Liquid Extraction

The powdered plant material was extracted with aqueous solvents, which contained 1% (*w*/*v*) citric acid, adjusted to the desired pH, and various amounts of either β-CD, HP-β-CD, or m-β-CD. The pH, as well as the exact amount of each cyclodextrin and powdered material, were defined by the experimental design (see paragraph 2.4). CDs were incorporated into the aqueous solutions prior to extractions. The extractions were performed under continuous magnetic stirring at 400 rpm, at ambient temperature (22 ± 1 °C), at a final volume of 25 mL, in glass vials, for 180 min. After each extraction, centrifugation of samples was carried out at 10,000× *g* and the clear supernatant was utilized for further analyses.

2.4. Experimental Design

The scope of the investigation was to study the effect of cyclodextrin (β-CD, HP-β-CD, m-β-CD) concentration (C_{CD}), pH, and liquid-to-solid ratio ($R_{L/S}$) on the performance of aqueous extraction of polyphenols from *S. fruticosa*. To accomplish this, a response surface methodology was employed, using the Box–Behnken experimental design including three central points, which enables determination of the first- and second-order coefficients of the mathematical model with high reliability [9]. Total polyphenol yield (Y_{TP}) was the screening response and codification of the variables chosen (C_{CD}, pH, $R_{L/S}$) between −1 (lower limit) and 1 (upper limit), and was performed as follows:

$$x_i = \frac{X_i - X_0}{\Delta X_i},\ i = 1, 2, 3 \quad (1)$$

where x_i is the dimensionless value of the independent variable i, and X_i is its actual value. X_0 represents the actual value of variable i at the central point of the design, and ΔX_i is the step change of X_i, corresponding to a change by a unit of the dimensionless value (Table 1). The ranges used for the independent variables were chosen on the basis of critical evaluation of literature data. The significance of the model, each polynomial coefficient, and the model coefficient R^2 were acquired by performing analysis of variance (ANOVA). On the basis of this analysis, insignificant dependent terms ($p > 0.05$) were not included in the mathematical equations (models). The desirability function enabled the determination of the optimal extraction conditions for maximizing Y_{TP} and visualization of the independent variable effect on Y_{TP} was delivered as 3D response surface plots. Model validation was

done by comparing predicted and experimental response values, after carrying out experiments under optimal extraction conditions.

Table 1. Process variables (actual and coded levels) considered for the design of experiment.

Independent Variables	Code Units	Coded Variable Level		
		−1	0	1
C_{CD} (%, *w/v*)	X_1	0.60	1.00	1.40
$R_{L/S}$ (mL g^{-1})	X_2	20	60	100
pH	X_3	3	5	7

2.5. Determination of Total Polyphenols (TP)

A method reported elsewhere was employed [10]. Aliquot of 0.5 mL of extract was combined with an equal volume of methanol containing 1% (*w/v*) trichloroacetic acid in a 1.5 mL Eppendorf tube. A volume of 0.02 mL of this mixture was then combined with 0.05 mL Folin–Ciocalteu reagent and 0.78 mL distilled water. After a 2 min reaction at room temperature, 0.15 mL Na_2CO_3 solution (20% *w/v*) was added and the samples were left to react for 60 min, in the dark. The absorbance at 740 nm was then obtained, using appropriate blank, and the concentration in total polyphenols (C_{TP}, mg L^{-1}) was calculated from a calibration curve, constructed with gallic acid as standard. Total polyphenol yield (Y_{TP}) was estimated by the following equation and expressed as mg gallic acid equivalents (GAEs) g^{-1} dry mass (dm):

$$Y_{TP}\left(\text{mg GAE g}-^{1}\right) = \frac{C_{TP} \times V}{dm} \quad (2)$$

where V is the volume (in L) of extraction and dm the dry mass of the plant material (in g).

2.6. Antiradical Activity (A_{AR}) Measurement

For the determination A_{AR}, a DPPH assay was used [10]. Samples were diluted 1:20 with methanol before each analysis. A volume of 0.025 mL of diluted sample was mixed with 0.975 mL of DPPH solution (100 µM in methanol) and the absorbance was immediately recorded at 515 nm ($A_{515(i)}$). The mixture was allowed to react for 30 min and then recording of absorbance at 515 nm was repeated ($A_{515(f)}$). A_{AR} was calculated as follows:

$$A_{AR} = \frac{C_{DPPH}}{C_{TP}} \times \left(1 - \frac{A_{515(f)}}{A_{515(i)}}\right) \times Y_{TP} \quad (3)$$

where C_{DPPH} is the DPPH concentration (µM) and C_{TP} is the and total polyphenol concentration (mg L^{-1}) in the reaction mixture; $A_{515(f)}$ is the A_{515} at $t = 30$ min and $A_{515(i)}$ the A_{515} at $t = 0$; and Y_{TP} is the total polyphenol yield (mg g^{-1}) of the extract. A_{AR} was expressed as µmol DPPH g^{-1} dm.

2.7. Ferric-Reducing Power (P_R) Determination

P_R of the extracts was assayed as described previously [10]. A volume of 0.05 mL of extract, diluted 1:20 with methanol, was combined with 0.05 mL $FeCl_3$ (4 mM in 0.05 M HCl) and the mixture was incubated in a thermostated water bath, set at 37 °C, for 30 min. After incubation, 0.9 mL TPTZ solution (1 mM in 0.05 M HCl) was added, and after exactly 10 min, the absorbance at 620 nm was measured. P_R was determined from an ascorbic acid calibration curve (50–300 µM) and given as µM ascorbic acid equivalents (AAEs) g^{-1} dm.

2.8. High-Performance Liquid Chromatography (HPLC)

The equipment was a FinniganMAT P4000 pump and a UV6000LP diode array detector (Thermo Scientific, Waltham, MA, USA). Chromatography was performed with a 10 µL injection loop, on a

Superspher RP-18 column, 125 mm × 2 mm, 4 μm, maintained at 40° C. The eluents were (A) and (B) were 2.5% acetic acid and methanol, respectively. The elution program used was as follows: 0 min, 100% A; 22 min, 65% A; 32 min, 65% A; 60 min, 0% A; 65 min, 0% A. The flow rate was 0.3 mL min^{-1}.

2.9. Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS)

The chromatograph was a TSQ Quantum Access LC/MS/MS, with a surveyor pump (Thermo Scientific, Wallthan, MA, USA), interfaced by XCalibur 2.1, TSQ 2.1 software. Analyses were carried out on a Superspher RP-18 column, 125 mm × 2 mm, 4 μm, at 40 °C, with 10 μL injection volume and a flow rate of 0.3 mL min^{-1}. Eluents and elution program were as described above. Mass spectra were acquired in negative ionization mode, with the following settings: sheath gas pressure, 30 mTorr; capillary temperature, 300 °C; collision pressure at 1.5 mTorr; auxiliary gas pressure, 15 mTorr. Quantification was accomplished with external standard methodology, using a luteolin 7-*O*-glucoside (5–1500 μg L^{-1}, R^2 = 0.9982) and a rosmarinic acid (50–3000 μg L^{-1}, R^2 = 0.9985) calibration curve. The standards were dissolved in HPLC grade methanol and stored at −17 °C.

2.10. Statistical Analysis

All extraction procedures were accomplished at least twice, and all determinations were carried out in triplicate. Values were given as average values ± standard deviation. All statistics pertaining to the experimental design were provided by JMP™ Pro 13. Linear and non-linear correlations, as well as curve fittings, were performed at least at a 95% significance level, with SigmaPlot™ 12.5.

3. Results and Discussion

3.1. Optimisation of the Extraction Performance

The process implemented was designed to appraise the effect of three crucial extraction variables, C_{CD}, $R_{L/S}$, and pH, and to identify possible synergistic functions between them. Evaluation of the fitted model and the suitability of response surface were based on the ANOVA and lack-of-fit test (Table 2), by considering the closeness of the predicted and measured values (Table 3). The second-degree polynomial equations (mathematical models), considering only the significant terms, are presented in Table 4, along with the square correlations coefficients (R^2) of the models, which are indicators of the total variability around the mean determined by the model. Because all total R^2 of the models were equal or higher than 0.97, and the *p*-value for lack of fit (assuming a confidence interval of 95%) was highly significant for all models, it can be argued that equations exhibited excellent fit to the experimental data. The contour plots constructed on the basis of the models, which are presented on a comparative arrangement in Figure 2, provide an at-a-glance image of how the experimental variables affected response (Y_{TP}), but also illustrate the differences between the three CDs used.

For the extraction with β-CD, C_{CD} was found to exert a non-significant effect, suggesting that any shift in C_{CD} within the range tested cannot impact Y_{TP}. The same was observed for HP-β-CD, but for m-β-CD, this variable was highly significant (p = 0.0047). This outcome strongly indicated that the nature of the CD used may play a key role in extraction. On the other hand, no cross term between m-β-CD concentration and either $R_{L/S}$ or pH was significant, showing that combined effects did not occur. Contrary to those, for the extractions performed with any CD, both $R_{L/S}$ and pH were significant.

Quadratic effects of these variables were also significant for the extractions with β-CD and m-β-CD, but for HP-β-CD, a significant quadratic effect was seen only for pH. Moreover, for HP-β-CD and m-β-CD, cross terms of $R_{L/S}$ and pH were significant too, demonstrating that combinations of these two variables may have either a negative (HP-β-CD) or positive (m-β-CD) influence on the extraction yield.

The desirability function (Figure 3) enabled the estimation of the optimal predicted response for each CD tested (Table 5). The Y_{TP} achieved with HP-β-CD and m-β-CD were identical and significantly higher than that obtained with β-CD ($p < 0.05$). This finding highlighted the prominent role of the nature of the CD used for the extraction. In support of this are pertinent results on

the extraction of olive pomace polyphenols, where HP-β-CD exhibited superior extraction capacity compared with either m-β-CD or γ-CD [11]. Data on polyphenol extraction from pomegranate fruit were in the same line [12], stressing the superiority of HP-β-CD against β-CD as a polyphenol extraction booster. In opposition, anthocyanin extraction was more efficient with β-CD rather than HP-β-CD [13]. Such discrepancies might emerge from the different encapsulating capacity of the CDs used towards structurally unrelated polyphenolic constituents. Indeed, examinations with pure polyphenols (catechin) demonstrated a more efficient encapsulation with β-CD than HP-β-CD or m-β-CD [14]. Therefore, the higher-performance extraction of *S. fruticosa* polyphenols observed with m-β-CD might reflect the manifestation of such phenomena. Given that the modelling performed revealed significant effect of C_{CD} only for m-β-CD, then it could be postulated that m-β-CD interacted more strongly with *S. fruticosa* polyphenols than β-CD or HP-β-CD within the C_{CD} limits tested.

Table 2. Statistical data associated with the mathematical models, built using response surface methodology. m-β-CD, methyl β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin.

Term	Standard Error	t Ratio	Probability > t	Sum of Squares	*F* Ratio
β-CD					
Intercept	0.846624	51.50	<0.0001 *	6.31901	-
C_{CD}	0.518449	1.71	0.1471	239.91451	2.9386
$R_{L/S}$	0.518449	10.56	0.0001 *	252.90005	111.5718
pH	0.518449	−10.84	0.0001 *	12.14523	117.6107
C_{CD} $R_{L/S}$	0.733198	−2.38	0.0634	0.11560	5.6481
C_{CD} pH	0.733198	−0.23	0.8258	0.65610	0.0538
$R_{L/S}$ pH	0.733198	−0.55	0.6045	1.93408	0.3051
C_{CD} C_{CD}	0.763136	−0.95	0.3865	53.14168	0.8994
$R_{L/S}$ $R_{L/S}$	0.763136	−4.97	0.0042 *	82.82608	24.7134
pH pH	0.763136	−6.21	0.0016 *	6.31901	38.5181
Lack-of-Fit			0.1067	9.972175	8.5298
HP-β-CD					
Intercept	1.05319	44.02	<0.0001 *	1.88180	-
C_{CD}	0.644945	0.75	0.4859	298.77901	0.5655
$R_{L/S}$	0.644945	9.48	0.0002 *	130.81531	89.7874
pH	0.644945	−6.27	0.0015 *	3.18623	39.3119
C_{CD} $R_{L/S}$	0.912089	0.98	0.3728	6.94323	0.9575
C_{CD} pH	0.912089	1.44	0.2082	22.94410	2.0865
$R_{L/S}$ pH	0.912089	−2.63	0.0468 *	1.93186	6.8950
C_{CD} C_{CD}	0.949333	0.76	0.4805	9.20776	0.5806
$R_{L/S}$ $R_{L/S}$	0.949333	−1.66	0.1571	80.32413	2.7671
pH pH	0.949333	−4.91	0.0044 *	1.88180	24.1386
Lack-of-Fit			0.1153	15.332875	7.8313
m-β-CD					
Intercept	0.547155	87.27	<0.0001 *	34.56961	
C_{CD}	0.273577	7.60	0.0047 *	195.89960	57.7357
$R_{L/S}$	0.335062	18.09	0.0004 *	193.40255	327.1775
pH	0.335062	−17.97	0.0004 *	0.18923	323.0072
C_{CD} $R_{L/S}$	0.386897	−0.56	0.6133	3.27610	0.3160
C_{CD} pH	0.386897	2.34	0.1013	17.09663	5.4715
$R_{L/S}$ pH	0.547155	5.34	0.0128 *	6.91763	28.5536
C_{CD} C_{CD}	0.47385	3.40	0.0425 *	20.80413	11.5533
$R_{L/S}$ $R_{L/S}$	0.47385	−5.89	0.0097 *	79.95325	34.7456
pH pH	0.47385	−11.56	0.0014 *	34.56961	133.5322
Lack-of-Fit			0.8547	0.4840687	0.1844

Asterisk (*) denotes statistically significant value, at least at a 95% significance level.

Table 3. Experimental design points and the corresponding predicted and measured Y_{TP} values for the extractions carried out with each of the CDs used. GAE, gallic acid equivalent.

Design Point	Independent Variables			Response (Y_{TP}, mg GAE g^{-1} dw)					
	C_{CD} (X_1)	$R_{L/S}$ (X_2)	pH (X_3)	β-CD		HP-β-CD		m-β-CD	
				Measured	Predicted	Measured	Predicted	Measured	Predicted
1	−1	−1	0	30.74	30.97	39.03	39.80	38.16	38.21
2	−1	1	0	46.97	45.41	49.94	50.24	51.03	50.77
3	1	−1	0	34.68	36.24	39.29	38.99	42.54	42.80
4	1	1	0	43.94	43.70	53.77	53.00	54.54	54.49
5	0	−1	−1	35.63	34.81	34.64	35.66	42.68	42.37
6	0	−1	1	25.35	24.38	33.85	32.36	26.50	24.47
7	0	1	−1	45.60	46.57	51.18	52.67	48.64	48.64
8	0	1	1	33.70	34.52	40.81	39.79	42.13	42.44
9	−1	0	−1	42.12	42.70	49.09	47.30	48.47	48.73
10	1	0	−1	45.56	44.82	46.35	45.63	51.03	51.08
11	−1	0	1	31.06	31.80	35.86	36.58	34.93	34.88
12	1	0	1	33.82	33.24	38.39	40.18	41.11	40.85
13	0	0	0	43.93	43.60	45.72	46.36	48.56	47.75
14	0	0	0	43.99	43.60	46.10	46.36	48.50	47.75
15	0	0	0	42.88	43.60	47.27	46.36	46.94	47.75

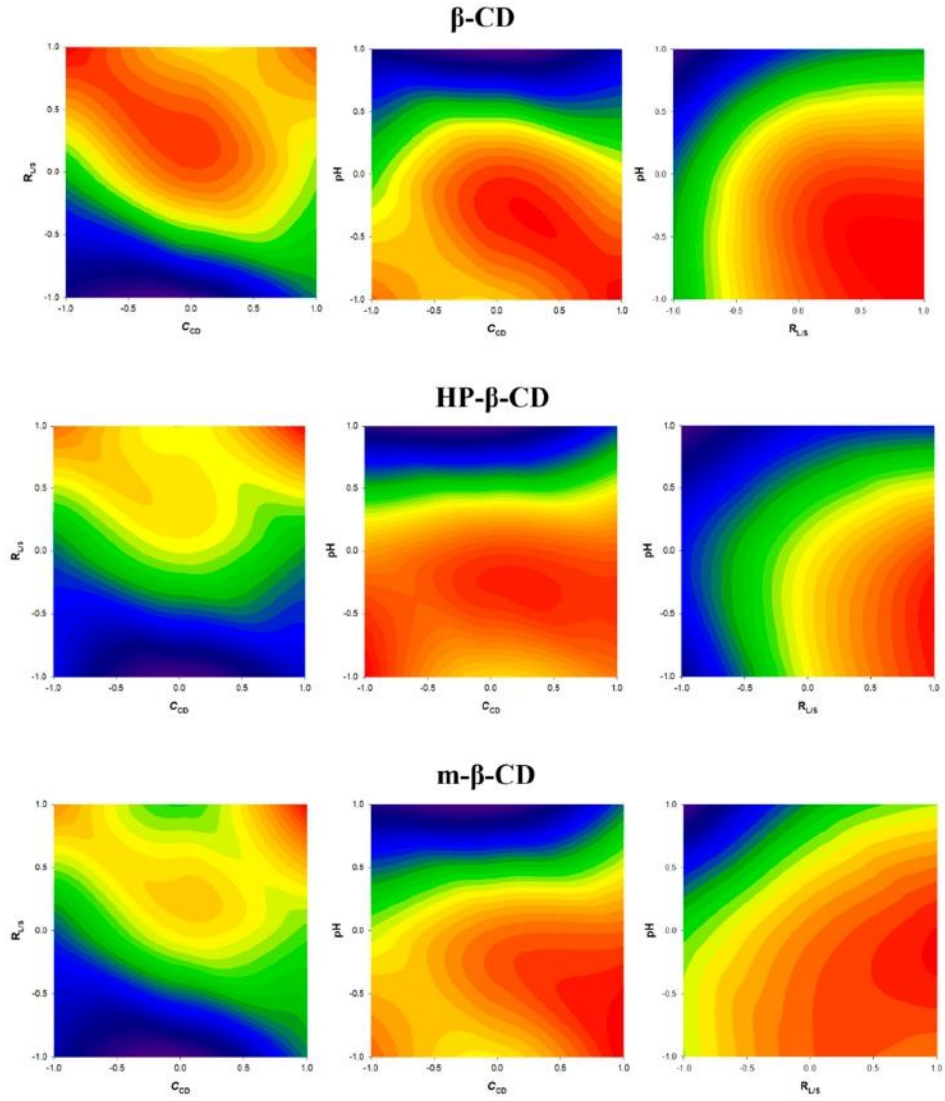

Figure 2. Contour graphs presenting the effect of simultaneous variation of independent variables on the response. Assignments: β-CD, β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin; m-β-CD, methyl β-cyclodextrin.

Table 4. Mathematical models and associated statistics derived from the experimental design.

Cyclodextrin	2nd Order Polynomial Equations	R^2	*p*
β-CD	$43.60 + 5.48X_2 - 5.63X_3 - 3.79X_2^2 - 4.74X_3^2$	0.98	0.0006
HP-β-CD	$46.36 + 6.11X_2 - 4.04X_3 - 2.39X_2X_3 - 4.66X_3^2$	0.97	0.0025
m-β-CD	$47.75 + 2.08X_1 + 6.06X_2 - 6.02X_3 + 2.92X_2X_3 - 2.79X_2^2 - 5.48X_3^2$	1.00	0.0024

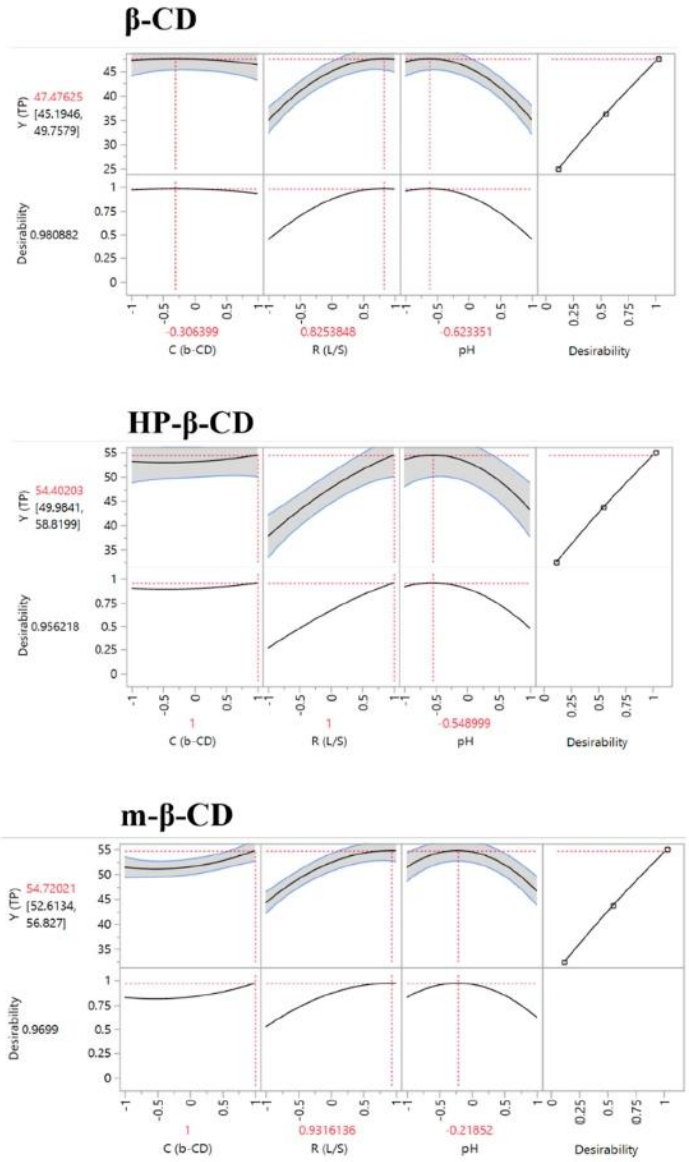

Figure 3. Desirability function for each of the CDs tested, displaying optimal conditions and maximum predicted response values. Assignments: m-β-CD, methyl β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin; β-CD, β-cyclodextrin.

Table 5. Values of the optimal predicted conditions and maximum predicted Y_{TP} (± sd) for *S. fruticosa* polyphenol extraction by the CDs tested.

Cyclodextrin	Maximum Predicted Response (Y_{TP}, mg GAE g^{-1} dw)	Optimal Conditions		
		C_{CD} (*w/v*, %)	$R_{L/S}$ (mL g^{-1})	pH
β-CD	47.48 ± 2.29	0.88	93	3.75
HP-β-CD	54.40 ± 4.42	1.40	100	3.90
m-β-CD	54.72 ± 2.11	1.40	94	4.54

For all CDs, optimum $R_{L/S}$ varied closely within 93–100 mL g^{-1} (Table 5). This outcome showed that the influence exerted by $R_{L/S}$ on the extraction performance was not significantly affected by the structure of CD. The magnitude of $R_{L/S}$ is related with the concentration gradient between the liquid phase (extraction medium) and the surface of the solid particle, which is directly involved in mass transfer. If $R_{L/S}$ is below a certain limit, then the equilibria established may not favor fast diffusion of the solute during extraction, owing to non-negligible resistance to mass transfer [15]. Several examinations on polyphenol extraction from plant tissues using conventional organic solvents suggested $R_{L/S}$ optima between 81 [16] and 100 mL g^{-1} [17–19]. Considering that the average $R_{L/S}$ value in this study was 96 mL g^{-1}, it could be argued that an aqueous medium containing any of the CDs assayed would behave as a common solvent in this regard.

The statistically significant effects of pH revealed by the models for the extraction with any of the CDs assayed pointed emphatically to the role of the pH in the extraction performance. For all CDs, the optimal pH was below 5, which evidenced that extractions were favoured at acidic pH. One possible reason for this might be related to the ionisation of the phenolic hydroxyl groups, which possess weak acidity. Assuming that encapsulation of polyphenols within the CD cavity is the main effect that enhances extraction, then polyphenol dissociation would increase their polarity, leading to weaker interactions with CD cavity, which is hydrophobic. As dissociation would increase at a higher pH, it would be likely that suppression of dissociation at pH < 5 would maintain polyphenols in their molecular (non-dissociated) form, hence promoting more powerful polyphenol–CD interactions. In support of such a hypothesis were results on naproxen interactions with β-CD, where increasing pH was demonstrated to provoke instability on the inclusion complex, a fact ascribed to lower affinity of charged drug for the hydrophobic β-CD cavity [20].

Although accurate determination has shown that pK_a may lie well above 7 for several substituted phenolics [21], for some flavonols such as quercetin, which are frequently encountered in plant tissues, pK_{a1} may vary within 5.06 to 7.36 [22]. In any case, at pH > 5, even limited dissociation of the most acidic phenolic hydroxyls could occur, provoking a significant increase in polyphenol polarity. This issue was also addressed in previous studies on polyphenol extraction with water/ethanol mixtures [23–25], where optimal extraction pH for total polyphenols was always <5. In these cases, increased extraction yield was ascribed to higher solubility of non-dissociated polyphenols in ethanol-containing solvents and increased polyphenol stability at acidic pH.

Variations in C_{CD} concentration within the limits tested for β-CD and HP-β-CD were shown to exert non-significant impact on Y_{TP}, but it was not clear whether the presence of any CD used could affect Y_{TP}. To examine this, extractions were performed with each CD under optimised conditions, as well as with aqueous solutions under the same $R_{L/S}$ and pH, without the addition of CD (Table 6). In every case, it was demonstrated that addition of CDs provoked significantly higher Y_{TP}, highlighting the importance of the CDs used as aqueous extraction boosters. The highest difference in Y_{TP} was found for m-β-CD (22.06%), followed by HP-β-CD (19.32%) and β-CD (11.28%).

Table 6. The effect of each of the CDs tested on the extractability of polyphenols from *S. fruticosa*, compared with equally buffered deionized water, under optimal $R_{L/S}$.

Extraction Medium	Y_{TP} (mg GAE g^{-1} dw)	Extraction Conditions		
		C_{CD} (*w/v*, %)	$R_{L/S}$ (mL g^{-1})	pH
β-CD	45.75 ± 1.11	0.88	93	3.75
Buffered dH_2O	40.59 ± 1.01	-	93	3.75
HP-β-CD	50.52 ± 1.19	1.40	100	3.90
Buffered dH_2O	40.76 ± 1.02	-	100	3.90
m-β-CD	54.25 ± 1.35	1.40	94	4.54
Buffered dH_2O	42.28 ± 1.05	-	94	4.54

3.2. Extraction Kinetics and Temperature Effects

To appraise the temperature effects on polyphenol recovery, kinetics was traced using all three CDs over a range of 40 to 80 °C (Figure 4). The model that could effectively describe the patterns recorded was second-order kinetics [26]:

$$Y_{TP(t)} = \frac{Y_{TP(s)}^2 kt}{1 + Y_{TP(s)} kt} \quad (4)$$

where $Y_{TP(t)}$ and $Y_{TP(s)}$ represent the TP yield at any time t and at equilibrium (saturation), respectively. k is the second-order extraction rate constant. When t approaches 0, the initial extraction rate, h, given as $Y_{TP(t)}/t$, is defined as follows:

$$h = kY_{TP(s)}^2 \quad (5)$$

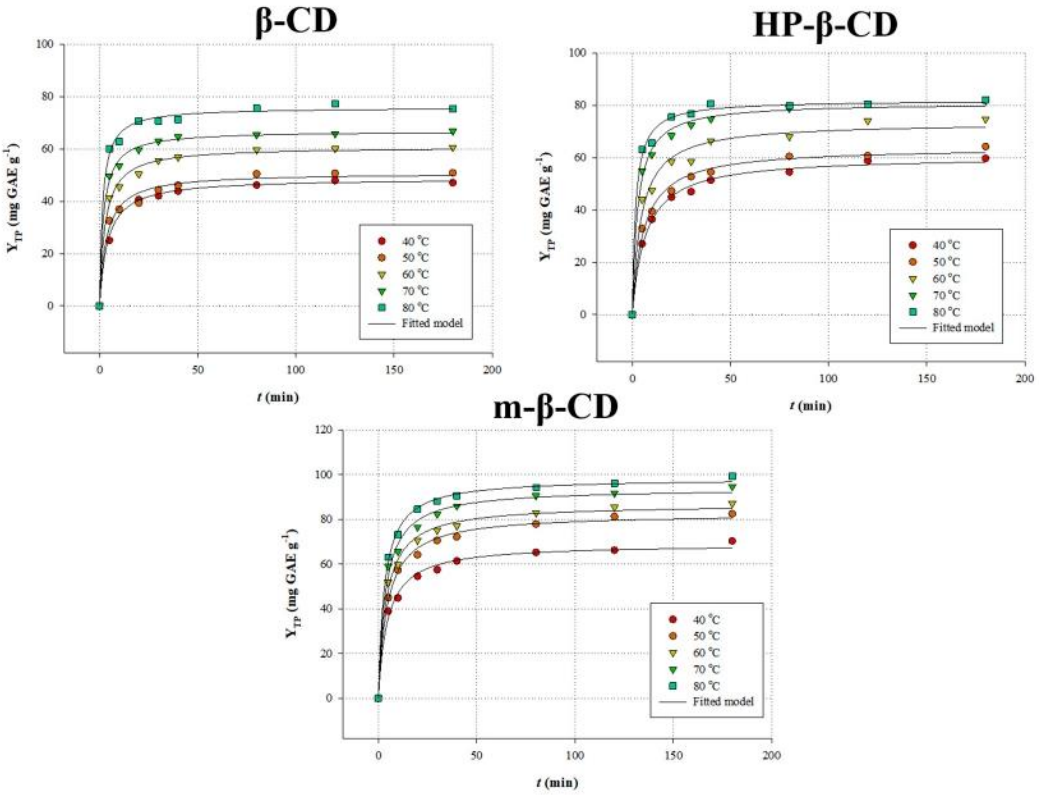

Figure 4. Kinetics of polyphenol extraction from *S. fruticosa*, with each of the CDs tested, within a range from 40 to 80 °C. Extractions were performed under optimized conditions. Assignments: m-β-CD, methyl β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin; β-CD, β-cyclodextrin.

In Table 7, the values determined for k, $Y_{TP(s)}$ and h, using SigmaPlot™ 12.5, can be seen. For all CDs, the three kinetic parameters exhibited an increase as a response to raising the temperature up to 80 °C.

The highest $Y_{TP(s)}$ was achieved with m-β-CD (98.39 mg GAE g^{-1} dm) at 80 °C, and it was only 5.3% lower than that achieved with 60% methanol (Figure 5). Both β-CD and HP-β-CD were significantly less effective, giving $Y_{TP(s)}$ 75.85 and 81.93 mg GAE g^{-1} dm, respectively. To further assess the impact of temperature, samples obtained at the end of each treatment (180 min) were also assayed for antioxidant activity (Figure 6). In line with $Y_{TP(s)}$, extracts obtained with m-β-CD exhibited the highest A_{AR} (1112.51 μmol DPPH g^{-1} dm), followed by HP-β-CD (836.17 μmol DPPH g^{-1} dm) and β-CD (824.08 μmol DPPH g^{-1} dm). The results for P_R were in concurrence, giving corresponding

values of 241.88, 210.72, and 185.74 μmol AAE g^{-1} dm. This outcome suggested that, using m-β-CD, polyphenol-enriched extracts with improved antioxidant characteristics may be produced at 80 °C.

Table 7. Kinetic parameters determined for the extraction of *S. fruticosa* polyphenols with the CDs tested. Extractions were accomplished under optimal C_{CD}, $R_{L/S}$, and pH.

T (°C)	Kinetic Parameters		
	k (×10^{-3}) (g mg^{-1} min^{-1})	*h* (mg g^{-1} min^{-1})	$Y_{TP(s)}$ (mg GAE g^{-1})
β-CD			
40	4.95	11.76	48.74
50	5.59	14.45	50.86
60	5.83	21.51	60.75
70	7.53	33.70	66.89
80	8.46	48.68	75.85
HP-β-CD			
40	2.43	8.91	60.53
50	2.71	11.09	63.92
60	3.02	16.30	73.41
70	4.40	28.84	80.92
80	6.92	46.47	81.93
m-β-CD			
40	2.55	14.66	68.99
50	2.64	17.99	82.56
60	2.90	21.79	86.71
70	2.95	25.97	93.89
80	3.28	31.76	98.39

The effect of temperature on *k* was better illustrated by establishing correlations between *k* and *T* (Figure 7). These correlations could be very effectively described using an exponential model [27]: $k = k_0$

$$k = k_0 + ae^{-bT} \quad (6)$$

where *k* corresponds to the second-order extraction rate and k_0 to a pre-exponential factor. In Table 8, the parameters k_0, a, and b, calculated by SigmaPlot™ 12.5, are given analytically. Extraction with m-β-CD displayed the lowest b value, which suggested that it was the least affected by temperature, as opposed to the extraction with HP-β-CD. This finding evidenced that m-β-CD provided the most effective and the least energy-demanding extraction of polyphenols. To ascertain this and obtain a tentative estimation of the barriers required for the extraction with each CD tested, the activation energy was determined as follows [28]:

$$ln\left(\frac{k_{ref}}{k}\right) = \left(-\frac{E_a}{R}\right)\left(\frac{1}{T} - \frac{1}{T_{ref}}\right) \quad (7)$$

where T_{ref} was chosen as the mean temperature of testing (60 °C) and T = 40 °C. k_{ref} and k were the corresponding second-order extraction rate constants. E_a is the activation energy (J mol^{-1}) and R the universal gas constant (8.314 J K^{-1} mol^{-1}). E_a thus estimated for the extraction with β-CD, HP-β-CD, and m-β-CD were 7.18, 9.50, and 5.64 kJ mol^{-1}, respectively. This finding did confirm that the extraction with m-β-CD was the least energy-demanding process.

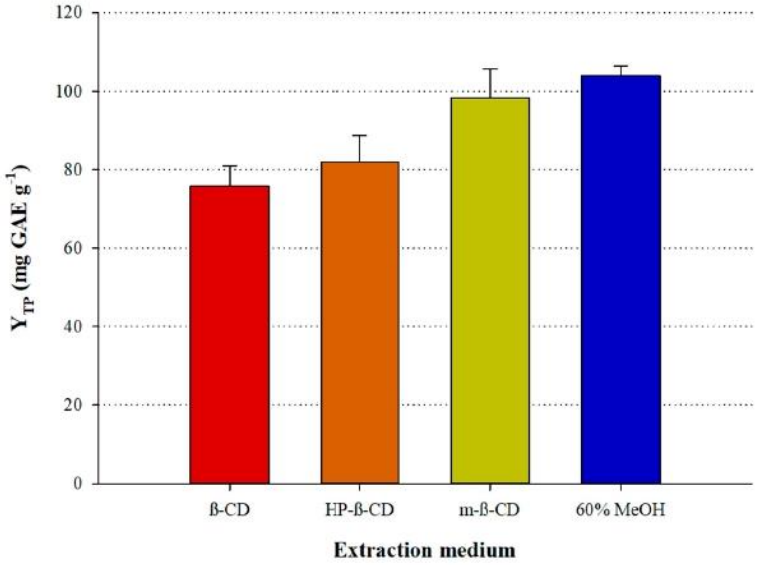

Figure 5. Plot showing Y_{TP} achieved using each of the CDs tested, under optimized conditions, at 80 °C, after 180 min.

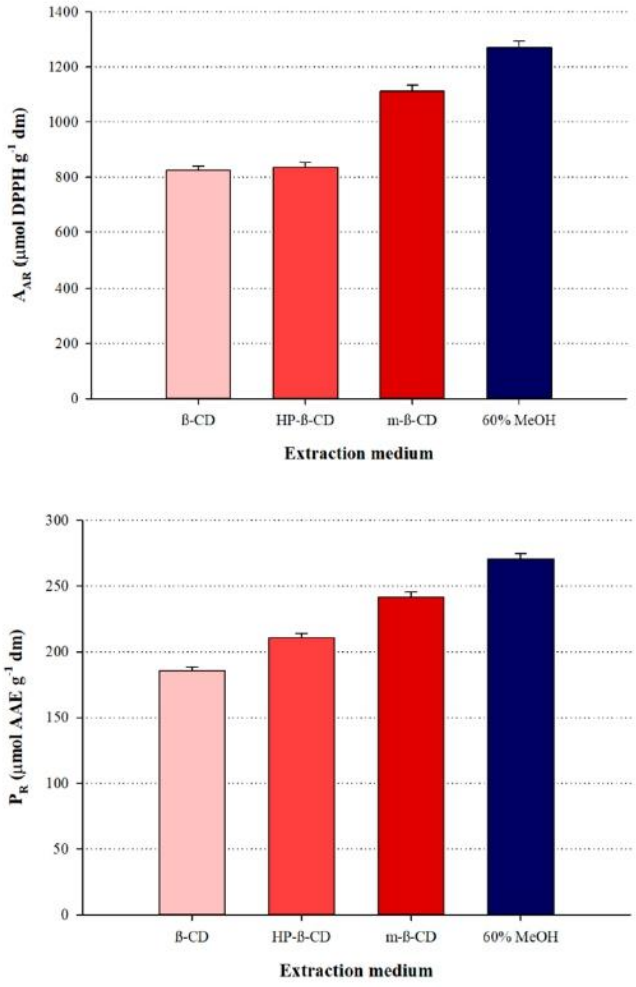

Figure 6. Diagram illustrating the A_{AR} and P_R of the extracts produced using each of the CDs tested, under optimized conditions, after 180 min, at 80 °C. Assignments: m-β-CD, methyl β-cyclodextrin; HP-β-CD, hydroxypropyl β-cyclodextrin; β-CD, β-cyclodextrin.

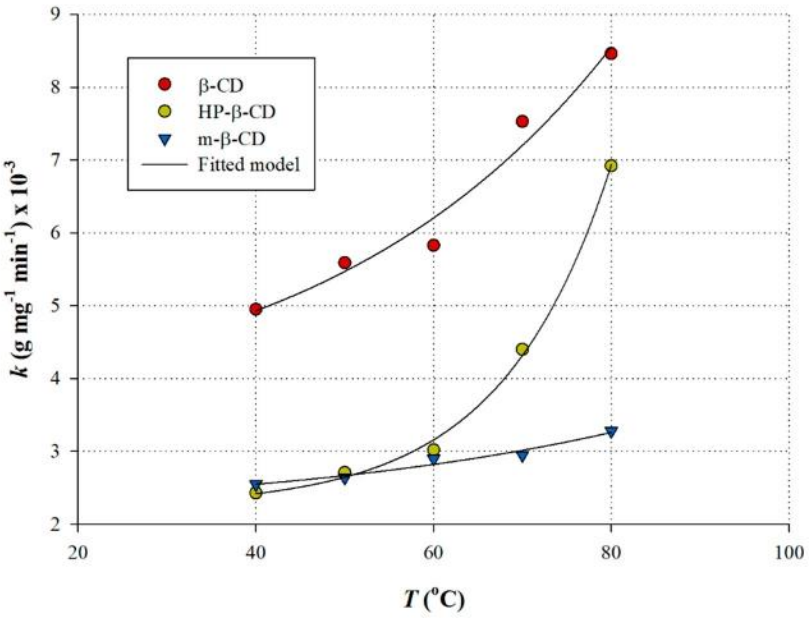

Figure 7. Non-linear regression between second-order extraction rates (k) and T.

Table 8. Fitting parameter values determined by correlating second-order extraction rates (k) with T.

CD	Parameter Estimates				
	k_0 ($\times 10^{-6}$)	a ($\times 10^{-5}$)	b	R^2	p
β-CD	3.409	0.4500	0.0305	0.97	0.0320
HP-β-CD	2.242	0.0068	0.0816	1.00	0.0022
m-β-CD	2.103	0.1727	0.0238	0.96	0.0351

3.3. Polyphenolic Profile

A chromatogram of a *S. fruticosa* extract, monitored at 350 nm, is given in Figure 8. The chromatograms corresponding to extracts obtained with either of the cyclodextrins tested did not display any significant difference (data not shown). In total, nine polyphenols could be reliably detected by carrying out LC/MS/MS, but for peak #1, no tentative structure could be proposed (Table 9). Peaks #2–9 were tentatively identified based on the information provided by previous studies [29,30].

Table 9. Spectral information pertaining to polyphenols detected in *S. fruticosa* extracts, obtained with either CD tested.

No	Rt (min)	UV/Vis (λ_{max})	$[M-H]^-$ (m/z)	Other Ions (m/z)	Tentative Identity
1	19.58	270, 340	593	-	Unknown
2	22.28	280, 344	477	301	6-Hydroxy luteolin 7-*O*-glucoside
3	24.38	256, 352	461	285	Luteolin 7-*O*-glucuronide
4	25.25	258, 348	593	285	Luteolin 7-*O*-rutinoside
5	25.82	270, 352	491	299	6-Methoxyluteolin 7-*O*-glucoside (nepitrin)
6	26.62	246, 316	359	161	Rosmarinic acid
7	27.50	264, 346	445	269	Apigenin 7-*O*-glucuronide
8	29.55	270, 352	475	299	6-Methoxyluteolin derivative
9	30.12	274, 332	461	299, 283	6-Methoxyluteolin derivative

To assess the efficiency of the CDs tested for polyphenol extraction, the two major constituents were considered, luteolin 7-*O*-glucuronide and rosmarinic acid, in order to minimize variations attributed to extraction. As can be seen in Table 10, extraction with m-β-CD afforded 7.7% higher luteolin 7-*O*-glucuronide yield compared with β-CD and 34.4% compared with HP-β-CD. On the other hand, β-CD was 10.1% more effective compared with m-β-CD and 13.3% compared with HP-β-CD in

extracting rosmarinic acid. This outcome suggested that the structural differences in CDs may account for selectivity towards different polyphenols. In all cases, it was observed that HP-β-CD was the least effective of the three CDs tested, and future studies pertaining to cyclodextrin-aided extraction of polyphenols such as flavonoid glycosides and rosmarinic acid, should consider m-β-CD as the most efficacious extraction booster.

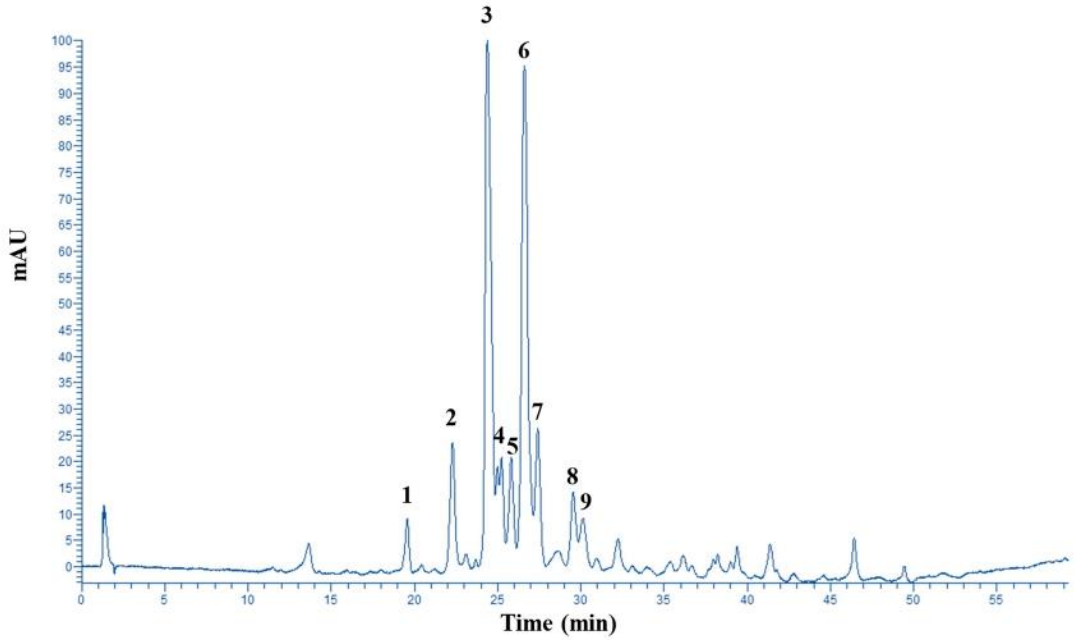

Figure 8. HPLC trace recorded at 330 nm, of a *S. fruticosa* extract, obtained with m-β-CD under optimized conditions, at 80 °C, after 180 min.

Table 10. Quantitative data on the recovery of major *S. fruticosa* polyphenols with the CDs tested, under optimal conditions.

Extract	Yield (mg g^{-1} dm)	
	Luteolin 7-*O*-glucuronide	**Rosmarinic Acid**
β-CD	3.35 ± 0.02	7.12 ± 0.00
HP-β-CD	2.38 ± 0.02	6.17 ± 0.10
m-β-CD	3.63 ± 0.03	6.40 ± 0.20

4. Conclusions

The current investigation examined in detail the aqueous extraction of bioactive polyphenols from the medicinal plant *S. frutocosa*, aided by the use of three different cyclodextrins. Following process optimization, m-β-CD was proven as the most efficient extraction booster, providing extracts with significant polyphenol yield and improved antioxidant characteristics. Extraction kinetics showed that (i) extraction performance and antioxidant activity may be even more enhanced at 80 °C and (ii) extraction with m-β-CD was the least energy demanding. LC/MS/MS analyses revealed that luteolin 7-*O*-glucuronide and rosmarinic acid were the predominant polyphenols in the extracts obtained with either CD, and that m-β-CD might exhibit higher affinity for luteolin 7-*O*-glucuronide, and β-CD for rosmarinic acid. The conclusions drawn may be of value in developing green extraction processes for effective polyphenol recovery, not only for *S. frutocosa*, but also other botanical species possessing similar polyphenolic composition. Furthermore, the selectivity issue concerning various CDs should be more thoroughly tested on plant matrices with variable polyphenolic composition, in order to study the effect of structural features on polyphenol extractability.

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

Nomenclature

A_{AR}	antiradical activity (μmol DPPH g^{-1})
C_{CD}	cyclodextrin (β-CD, HP-β-CD, m-β-CD) concentration (%, *w*/*v*)
dm	dry mass (g)
E_a	activation energy (kJ mol^{-1})
k_0	pre-exponential factor (g mg^{-1} min^{-1})
k	second-order extraction rate constant (g mg^{-1} min^{-1})
k_{ref}	second-order extraction rate constant at reference *T* (g mg^{-1} min^{-1})
P_R	reducing power (μmol AAE g^{-1})
R	universal gas constant (8.314 K^{-1} mol^{-1})
$R_{L/S}$	liquid-to-solid ratio (mL g^{-1})
t	time (min)
T	temperature (°C)
T_{ref}	reference *T* (°C)
Y_{TP}	yield in total polyphenols (mg GAE g^{-1})

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Hydroglycerolic Solvent and Ultrasonication Pretreatment: A Green Blend for High-Efficiency Extraction of *Salvia fruticosa* Polyphenols

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Abstract: *Salvia fruticosa* Miller, also known as Cretan or Greek sage, is a medicinal plant with significant biological properties, which are largely ascribed to its polyphenolic composition, but there is to-date a scarcity of green and sustainable processes for efficient polyphenol extraction from this plant. The objective of this study was the implementation of an extraction process that would combine a green solvent based on glycerol, a biodiesel industry by-product, and ultrasonication pretreatment. Ultrasonication for 40 min followed by stirred-tank extraction was shown to provide significantly higher total polyphenol yield than mere stirred-tank extraction, while kinetics indicated 50 °C as the most favorable temperature, with the yield being 92 mg gallic acid equivalents (GAE) per g dry mass. Comparison of this method with a previously developed one that used methyl β-cyclodextrin revealed that the extracts obtained had similar antioxidant activity, and yield in major polyphenols including luteolin 7-*O*-glucuronide and rosmarinic acid was virtually equal. The current process is proposed as a sustainable and effective methodology for the generation of polyphenol-enriched extracts from *S. fruticosa*, which could be used as effective food antioxidants/antimicrobials and/or cosmetic constituents.

Keywords: antioxidants; extraction kinetics; glycerol; green extraction; polyphenols; *Salvia fruticosa*; ultrasonication

1. Introduction

Consumer awareness and demand for functional food ingredients and health-promoting supplements have boosted a great development in botanical research [1] regarding new product design and enabled the launch of a wide spectrum of formulations [2] and cosmetic ingredients [3]. *Salvia* is a genus of the Lamiaceae family and embraces more than 800 species worldwide [4]. Numerous *Salvia* species are regarded as plants with significant bioactive properties, and they have been used for centuries as folk pharmaceuticals in many countries [5]. The therapeutic potential of *Salvia* plants has been largely ascribed to principal substances, including phenolic acids and terpenoids, but in *Salvia* species a large variety of flavonoids may also occur [6,7]. *S. fruticosa*, otherwise known as *S. triloba* (family: Lamiaceae), is a sage species native to the island of Crete (southern Greece). It is regarded as a plant of great biological value [8–10], yet there is to-date no green extraction process developed for

the generation of polyphenol-enriched extracts with high antioxidant activity, which could be used as active ingredients in food supplements, cosmetics, and pharmaceuticals.

The development of green processes aimed at producing polyphenol-enriched extracts from botanicals has been of great concern to researchers, and a number of eco-friendly, reproducible, low-cost and low-energy techniques are now acknowledged as more effective alternatives to traditional extraction methodologies [11]. However, one of the major ways to comply with the principles of green chemistry is to reduce the use of toxic, volatile organic solvents, and to encourage their replacement by novel, environmentally friendly liquids. In this framework, the selection of an appropriate solvent is of paramount importance to the sustainable character of an extraction method. The ideal candidate should display high extraction efficiency, low or no toxicity, low price, and availability, and it should be produced from recyclable resources, as opposed to petroleum-derived solvents [12,13].

Glycerol (glycerine or 1,2,3-propanetriol) is a bio-liquid considered a by-product of the biodiesel industry, which is generated at about 10% by weight of the starting material (triacylglycerols) [14]. Although glycerol is a well-established sustainable solvent for various chemical processes [14–16], its use as a green solvent for effective polyphenol extraction has been introduced only within the last six years [17]. Ever since, several studies have demonstrated glycerol/water mixtures as high-performing extraction media for polyphenol recovery from various plant matrices [18–26]. This being the case, the current project was undertaken to thoroughly examine the extraction of *S. fruticosa* polyphenolic antioxidants using green glycerol/water mixtures, combined with ultrasonication pretreatment. Major polyphenolic phytochemicals in the optimally produced extracts were tentatively identified with liquid chromatography-diode array-tandem mass spectrometry (LC/MS/MS).

2. Materials and Methods

2.1. Chemicals and Reagents

Methyl β-cyclodextrin chlorogenic acid (≥95%), luteolin 7-*O*-glucoside, and rosmarinic acid (96%) were from Sigma (St. Louis, MO, USA). Glycerol (99%) and ethanol (99.8%) were from Acros Organics (Geel, Belgium). Aluminium chloride hexahydrate and sodium acetate trihydrate were from Penta (Prague, Czeck Republic). 2,4,6-Tripyridyl-*s*-triazine (TPTZ, 99%), Folin–Ciocalteu reagent and ferric chloride hexahydrate were from Fluka (Steinheim, Germany). Anhydrous sodium carbonate was from Carlo Erba Reactifs (Val de Reuil, France). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), gallic acid, ascorbic acid, and rutin (quercetin 3-*O*-rutinoside) were from Aldrich (Steinheim, Germany). The solvents used for chromatographic analyses were HPLC grade.

2.2. Plant Material—Handling and Preparation

Salvia fruticosa, also known as *Salvia triloba* (Cretan or Greek sage), was purchased from a local store of certified botanicals (Chania, Greece) and further identified by the Mediterranean Plant Conservation Center (Chania, Greece). The material (250 g) consisted of dried aerial parts of the plant, and it was received in air-tight plastic packaging. Upon receipt, it was stored in a chamber of low humidity, in the dark, for no longer than a week. An amount of approximately 50 g of material was placed in a domestic blender, ground, and then sieved to yield a feed with an average particle diameter of 1.28 mm. This feed was transferred into plastic containers, stored at 7 °C, and used in all procedures.

2.3. Ultrasonication Pretreatment

An exact amount of 1 g of feed was mixed with 25 mL of solvent in a 50-mL round-bottom flask, and ultrasonicated in an ultrasonication bath (Sonorex Bandeline, Berlin, Germany) with the following settings: power, 120 W; acoustic energy density, 120 W L^{-1}; frequency, 100 Hz; temperature, 50 °C. Ultrasonication was performed for 5, 10, 20, 30, and 40 min.

2.4. Batch Stirred-Tank Solid–Liquid Extraction

This procedure was implemented after ultrasonication pretreatment. The solvents tested were deionized water as well as hydroglycerolic mixtures with glycerol proportions of 20%, 40%, 60%, and 80% (*w*/*v*). Extraction was undertaken in an oil bath at a constant temperature of 50 °C with stirring at 700 rpm for 150 min. Temperature regulation and stirring were provided by a heating magnetic stirrer (VELP Scientifica, Bohemia, NY, USA). After extraction, each sample was centrifuged at 10,000× *g* for 10 min, and the supernatant was used for all analyses performed afterwards.

2.5. Extraction Kinetics and Temperature Effects

Kinetics was examined by implementing the model previously proposed [27]:

$$Y_{TP(t)} = Y_{TP(0)} + \frac{Y_{TP(s)}t}{t_{0.5} + t} \quad (1)$$

$Y_{TP(t)}$ is the yield in total polyphenols at any time, t, $Y_{TP(s)}$ is the yield in total polyphenols at saturation (equilibrium), $Y_{TP(0)}$ is a fitting parameter, and $t_{0.5}$ represents the time at which $Y_{TP(t)} = \frac{Y_{TP(s)}}{2}$. According to this model, the initial extraction rate, h, and the second-order extraction rate, k, are given as:

$$h = \frac{Y_{TP(s)}}{t_{0.5}} \quad (2)$$

$$k = \frac{1}{Y_{TP(s)}\ t_{0.5}} \quad (3)$$

The effect of temperature on k was illustrated by performing non-linear regression between k and T. This correlation could be very effectively described using an exponential model [28]:

$$k = k_0 + ae^{-bT} \quad (4)$$

Terms k and k_0 correspond to the second-order extraction rate and a pre-exponential factor. Determination of the activation energy (E_a) of the process was computed as follows [29]:

$$ln\left(\frac{k}{k_{ref}}\right) = \left(-\frac{E_a}{R}\right)\left(\frac{1}{T} - \frac{1}{T_{ref}}\right) \quad (5)$$

T_{ref} and T are the reference temperature (K) and the temperature at which kinetics was traced, k_{ref} and k are the corresponding second-order extraction rate constants, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), and E_a is the activation energy (J mol^{-1}).

2.6. Determinations

Total polyphenol analysis was performed using a previously described Folin–Ciocalteu methodology [30]. Yield in total polyphenols (Y_{TP}) was given as mg gallic acid equivalents (GAE) per g dry mass (dm). Likewise, total flavonoids were determined with $CH_3COONa/AlCl_3$ reagent and given as mg rutin equivalents (RtE) per g dm [31]. The antiradical activity (A_{AR}) and the ferric-reducing power (P_R) were estimated as reported elsewhere [27], and results were expressed as µmol DPPH per g dm and µmol ascorbic acid equivalents (AAE) per g dm, respectively.

2.7. Chromatographic Determinations

Analyses were performed with a FinniganMAT P4000 pump equipped with a UV6000LP diode array detector (Thermo Scientific, Waltham, MA, USA), and a TSQ Quantum Access LC/MS/MS, coupled with a Surveyor pump (Thermo Scientific, Walltham, MA, USA), controlled by XCalibur 2.1, TSQ 2.1 software. Chromatography was run on a Superspher RP-18 column, 125 mm × 2 mm, 4 µm,

maintained at 40 °C, employing 10-µL injections. The eluents used were (A) 2.5% acetic acid and (B) methanol, at a flow rate of 0.3 mL min^{-1}. The elution program implemented was as follows: 0 min, 100% A; 22 min, 65% A; 32 min, 65% A; 60 min, 0% A; 65 min, 0% A. Mass spectra were acquired with negative ionization, with the following settings: sheath gas pressure 30 mTorr; collision pressure 1.5 mTorr; capillary temperature 300 °C; auxiliary gas pressure 15 mTorr. Quantification was carried out with external standard methodology, using a calibration curve of chlorogenic acid (50–1500 µg L^{-1}, R^2 = 0.9986), rosmarinic acid (50–3000 µg L^{-1}, R^2 = 0.9985), and luteolin 7-*O*-glucoside (5–1500 µg L^{-1}, R^2 = 0.9982). Standard solutions were prepared in HPLC grade methanol and stored in the freezer.

2.8. Statistical Analysis

Two repetitions were performed for each extraction and pretreatment process, and each determination was carried out in triplicate. Values given represent averages ± standard deviation. Linear correlations and kinetic model fitting were accomplished with SigmaPlot™ 12.5 (Systat Software Inc., San Jose, CA, USA). Distribution analysis, at least at a 95% significance level, was done with JMP™ Pro 13 (SAS, Cary, NC, USA).

3. Results and Discussion

3.1. Effect of Solvent Composition

Earlier examinations on the effect of glycerol/water proportion on polyphenol extraction employed rather low-glycerol mixtures, with glycerol percentage varying from 3.6% [32] to 9.3%–10% (*w*/*v*) [10,30,33]. However, later investigations showed that polyphenol extraction yield may increase linearly from 5% (*w*/*v*) onwards, the optimum being 20% (*w*/*v*) [12]. More thorough, single-factor studies including a wider range of glycerol/water proportions demonstrated that the optimum glycerol percentage may lie between 70% [34] and 90% (*w*/*v*) [35]. Optimum levels as high as 90% (*w*/*v*) have also been found by implementing response surface methodology [36,37]. Yet, significantly lower optimal levels of 20% (*w*/*v*) [16] and 32.5% (*w*/*v*) [19] have also been reported. Therefore, testing of the optimum glycerol/water proportion (C_{GL}) was performed over a range varying from 0% (deionized water) to 80% (*w*/*v*) glycerol (Figure 1). Proportions > 80% were not considered because high-glycerol mixtures are very viscous and particularly problematic to handle. The assay performed indicated that a mixture with C_{GL} of 60% (*w*/*v*) provided significantly higher ($p < 0.05$) total polyphenol extraction yield (Y_{TP}), which reached 66.92 ± 1.67 mg GAE g^{-1} dm. Thus, this solution was employed to perform further experimentation.

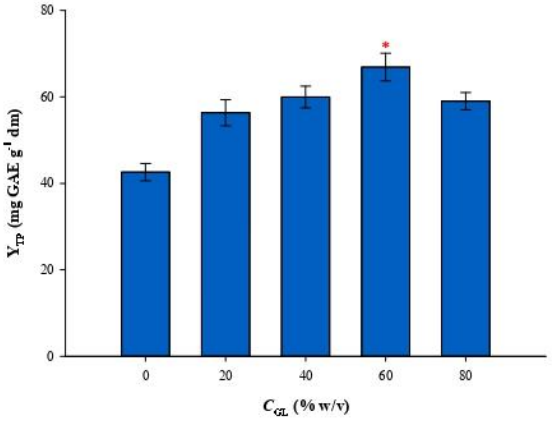

Figure 1. Assay performed to identify the optimum concentration of glycerol (C_{GL}) for *S. fruticosa* polyphenol extraction. Bars indicate standard deviation. Asterisk (*) denotes a statistically different value ($p < 0.05$).

3.2. *Effect of Ultrasonication Pretreatment*

The integration of ultrasonication as a pretreatment stage has been recently appraised, with the ultrasonication time considered ranging from 5 to 40 min [27,31,38]. On the basis of these data, the ultrasonication effect was tested within this time frame (Figure 2). As preliminary experiments showed that starting from 25 °C (room temperature), there may be an increase in temperature up to 45 °C after 40 min of ultrasonication, the assay temperature was set at 50 °C to eliminate variations arising from the ultrasonication effect. An ultrasonication temperature higher than 50 °C was not preferred to maximize the sonochemical benefit, in line with previous observations [39]. It has been proposed that ultrasound-assisted polyphenol extraction is not favored at temperatures higher than 50 °C because the collapse of cavitation bubbles, generated as a result of ultrasound irradiation, is more effective in low-vapor pressure solvents (such as glycerol/water mixtures) at lower temperatures. The collapse of cavitation bubbles is considered to enhance solute extraction because there is a release of a large amount of energy as a result of high temperature/high pressure involved in such a process. This in turn may contribute to disrupting the integrity of the solid particles, provoking an increased entrainment of solute in the liquid phase [40].

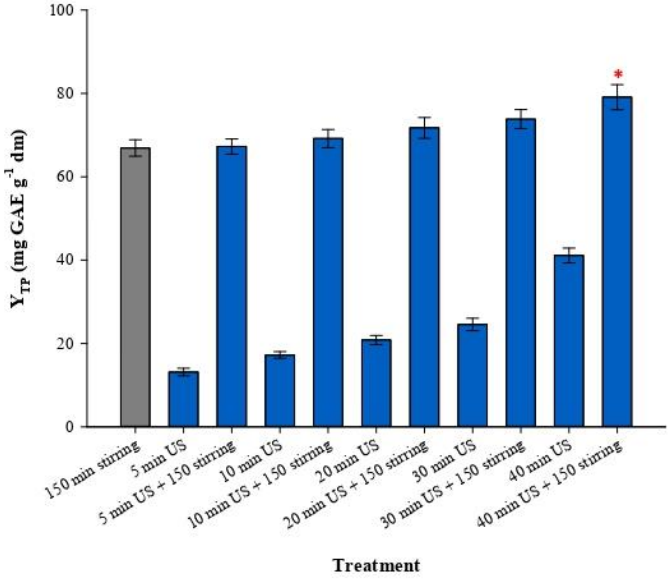

Figure 2. The effect of ultrasonication pretreatment on Y_{TP}, using 60% (*w*/*v*) glycerol/water mixture. Ultrasonication and subsequent stirred-tank extraction were performed at 50 °C. Bars indicate standard deviation. Asterisk (*) denotes a statistically different value ($p < 0.05$).

Changes in Y_{TP} displayed an increasing progression as a function of ultrasonication time, but significantly higher Y_{TP} ($p < 0.05$) was achieved with 40-min ultrasonication pretreatment. From 40 to 60 min, the yields achieved with ultrasonication alone were very similar (about 7% difference), whereas ultrasonication > 60 min resulted in a slight decline (about 8%) of the yield. The combination of pretreatment and a subsequent stirred-tank extraction afforded a Y_{TP} of 79.12 ± 1.98 mg GAE g^{-1} dm, which was 15% higher than that attained without pretreatment. This finding stressed emphatically the importance of ultrasonication pretreatment in boosting extraction efficiency. It is to be underlined that mere ultrasonication for 40 min gave a Y_{TP} of only 41.10 ± 1.03 mg GAE g^{-1} dm, which represented approximately just 52% of the Y_{TP} reached by combining ultrasonication pretreatment and stirred-tank extraction. This fact clearly demonstrated that ultrasonication was not effective as a standalone extraction mode. This was in absolute accordance with earlier results from similar studies on grape pomace [41] and elderflowers [31].

3.3. Extraction Kinetics and the Effect of Temperature

Previous studies showed that polyphenol extraction with hydroglycerolic solvents is significantly affected within a temperature spectrum ranging from 50 to 80 °C [35–37]. Thus, kinetics was traced at 50, 60, 70, and 80 °C to thoroughly investigate the influence of temperature (Figure 3).

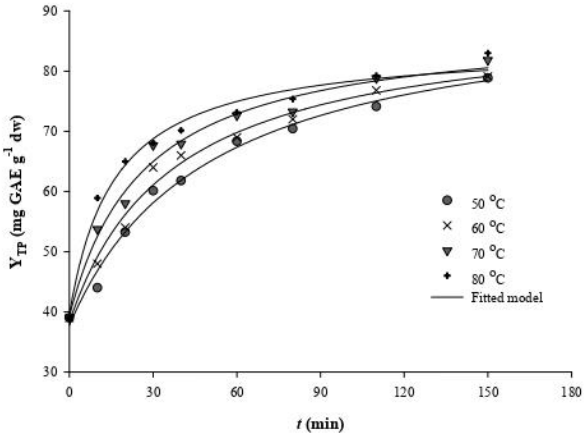

Figure 3. Kinetics of *S. fruticosa* polyphenol extraction, using 60% (*w*/*v*) glycerol/water mixture. Samples were pretreated with ultrasounds prior to stirred-tank extraction for 40 min at 50 °C.

Switching *T* from 50 to 80 °C resulted in progressive acceleration of extraction, as indicated by the increase in the second-order extraction rate, *k*, from 0.369 to 1.370 g mg^{-1} min^{-1} (Table 1). The pattern was similar for the initial extraction rate, *h*, which increased from 1.838 to 5.194 mg g^{-1} min^{-1}. The correlation of *k* with *T* was portrayed by an exponential model, as previously proposed [28], which showed excellent adjustment to the experimental data (Figure 4). The fitting parameter b equaled 0.0765, and it was significantly higher than 0.0238 determined for aqueous extraction of *S. fruticosa* polyphenols using methyl *β*-cyclodextrin [42]. This finding suggested that the stirred-tank extraction using hydroglycerolic solvent was more energy-demanding.

Table 1. Values of kinetic parameters determined for the extraction of *S. fruticosa* polyphenols, using 60% (*w*/*v*) glycerol/water mixture.

T (°C)	Kinetic Parameters			
	k ($\times 10^{-3}$) (g mg^{-1} min^{-1})	h (mg g^{-1} min^{-1})	$Y_{TP(s)}$ (mg GAE g^{-1})	$t_{0.5}$ (min)
50	0.369	1.838	92.00	50.06
60	0.528	2.400	89.27	37.19
70	0.768	3.278	87.91	26.82
80	1.370	5.194	84.53	16.27

To verify this assumption, the activation energy, E_a, was estimated using Equation (5), and the value found was 47.67 kJ mol^{-1}. This barrier was significantly higher than 5.64 kJ mol^{-1} determined for methyl *β*-cyclodextrin-assisted extraction [42], which confirmed the higher energy requirement. However, there is an important detail that should be taken into account. In this study, stirred-tank polyphenol extraction was applied after an ultrasonication regime of 40 min, during which a significant amount of readily extractable polyphenols was recovered (Figure 2). Thus, the E_a determined represented the barrier required to extract the residual and harder-to-extract polyphenols. Such a case has been recently investigated, and it was demonstrated that the E_a required to extract polyphenols from plant material after an ultrasonication pretreatment stage was higher than that corresponding to stirred-tank extraction without pretreatment [38].

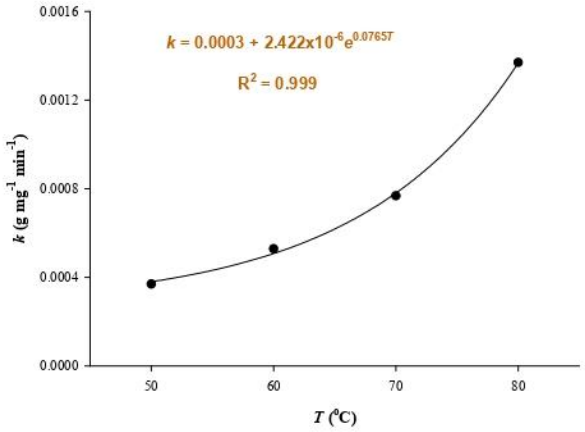

Figure 4. Non-linear regression between second-order extraction rate constant, *k*, and temperature, *T*.

YTP(s) displayed a declining trend and while its value was 92.00 mg GAE g−1 dm at 50 °C, it dropped to 84.53 mg GAE g−1 dm at 80 °C. However, distribution analysis indicated that this difference was non-significant ($p > 0.05$). This phenomenon has been previously reported for polyphenol extraction from onion solid wastes with hydroglycerolic mixture and attributed to polyphenol thermal instability [35]. In general, increases in T favor higher YTP because higher T usually entails higher polyphenol diffusion and solubility [43,44]. On the other hand, polyphenols are thermolabile molecules and in several cases T higher than 50 °C did not contribute to attaining increased YTP [45–47]. On the other hand, in a previous examination on cyclodextrin-aided aqueous extraction of S. fruticosa polyphenols, it was shown that polyphenol extraction yield increased constantly by raising T from 40 to 80 °C [42]. Such an effect could be attributed to the protective role of cyclodextrins against thermal degradation of polyphenols, as demonstrated by earlier studies [48].

3.4. Antioxidant Properties and Polyphenolic Profile

To test the effectiveness of the method developed, a comparison was carried out with another green method established previously [42], based on characteristics pertaining to polyphenol extraction yield and antioxidant activity (Table 2). Extraction with m-*β*-CD at 80 °C was proven more efficient with respect to Y_{TP}, as it afforded 108.14 ± 2.70 mg GAE g^{-1} dm, as opposed to extraction with the hydroglycerolic solvent, which gave by 22.5% lower Y_{TP} (83.86 ± 2.10 mg GAE g^{-1} dm). On the other hand, differences in Y_{TFn} and A_{AR} were marginal and non-significant ($p > 0.05$). On the contrary, the hydroglycerolic extract exhibited significantly higher P_R. The LC/DAD/MS/MS enabled the tentative identification of a series of polyphenolic phytochemicals (Figure 5, Table 3), based on spectral data reported earlier [42,49].

Table 2. Comparative evaluation of *S. fruticosa* extracts obtained with 60% (*w*/*v*) glycerol/water (GL) and methyl *β*-cyclodextrin (m-*β*-CD).

Extract	Y_{TP} (mg GAE g^{-1} dm)	Y_{TFn} (mg RtE g^{-1} dm)	A_{AR} (μmol DPPH g^{-1} dm)	P_R (μmol AAE g^{-1} dm)
m-*β*-CD	108.14 ± 2.70	53.62 ± 1.61	820.93 ± 16.42	590.66 ± 14.77
GL	83.86 ± 2.10	51.46 ± 2.57	817.58 ± 8.18	709.12 ± 17.73

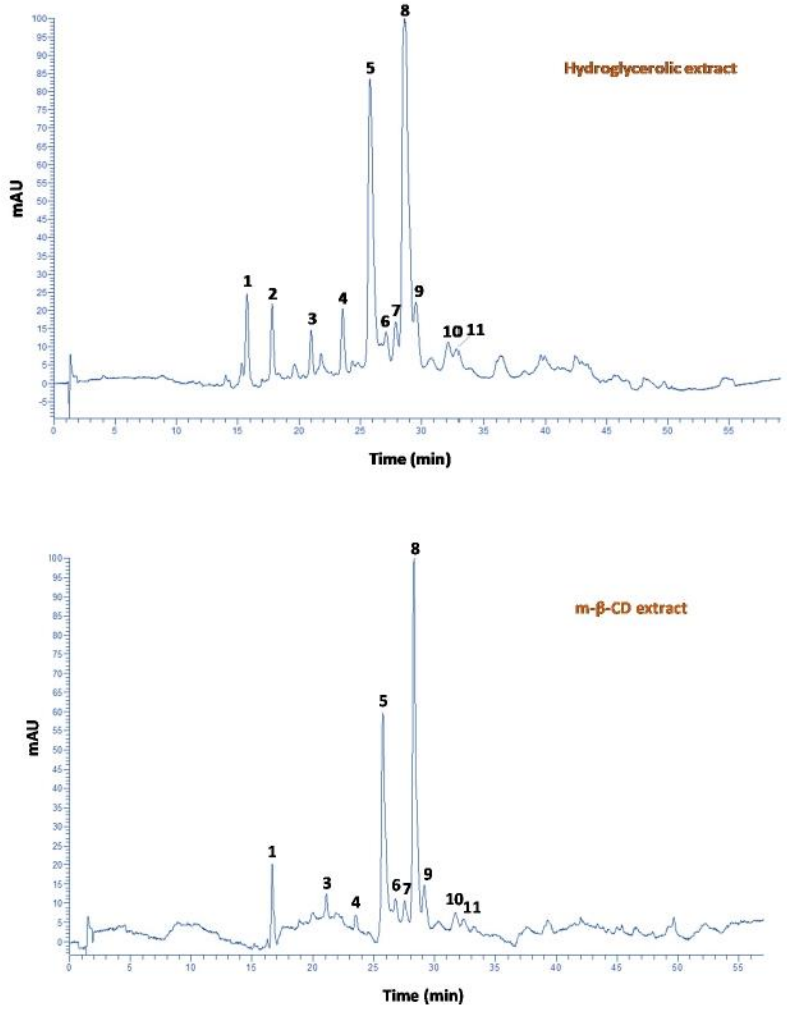

Figure 5. Typical HPLC traces of *S. fruticosa* polyphenol extracts, monitored at 330 nm. Extracts were produced with 60% (*w*/*v*) glycerol/water and methyl *β*-cyclodextrin (m-*β*-CD) at 80 °C. Peak assignment: 1, chlorogenic acid; 2, unknown; 3, unknown; 4, 6-hydroxy luteolin 7-*O*-glucoside; 5, luteolin 7-*O*-glucuronide; 6, luteolin 7-*O*-rutinoside; 7, 6-methoxyluteolin 7-*O*-glucoside (nepitrin); 8, rosmarinic acid; 9, apigenin 7-*O*-glucuronide; 10, 6-methoxyluteolin derivative; 11, 6-methoxyluteolin derivative.

Table 3. Spectral attributes used to tentatively identify major polyphenols in *S. fruticosa* extracts.

No	Rt (min)	UV-Vis (λ_{max})	$[M - H]^+$ (m/z)	Other Ions (m/z)	Tentative Identity
1	15.77	246, 318	353	179	Chlorogenic acid
2	17.40	248, 318	253	-	Unknown
3	21.00	270, 340	593	-	Unknown
4	23.57	280, 344	477	301	6-Hydroxy luteolin 7-*O*-glucoside
5	25.78	256, 352	461	285	Luteolin 7-*O*-glucuronide
6	27.12	258, 348	593	285	Luteolin 7-*O*-rutinoside
7	27.90	270, 352	491	299	6-Methoxyluteolin 7-*O*-glucoside (nepitrin)
8	28.65	246, 316	359	161	Rosmarinic acid
9	29.55	264, 346	445	269	Apigenin 7-*O*-glucuronide
10	32.15	270, 352	475	299	6-Methoxyluteolin derivative
11	32.82	274, 332	461	299, 283	6-Methoxyluteolin derivative

In order to better demonstrate the extraction capacity of the hydroglycerolic solvent, three major constituents were considered for quantitative analysis, namely chlorogenic acid, luteolin 7-*O*-glucuronide, and rosmarinic acid. Other minor polyphenols that were tentatively identified in the extracts were not considered because they occurred at significantly lower levels and differences

in their content might not be indicative for reliably assessing solvent extraction capacity. The results from the quantitative assay are analytically presented in Table 4. Compared to m-*β*-CD, extraction with the hydroglycerolic solvent gave a 37.5% higher yield in chlorogenic acid and a 0.57% higher yield in rosmarinic acid, but a 20.8% lower yield in luteolin 7-*O*-glucuronide. Overall, the difference in yield was only 7.4%, indicating that both extracting media performed equally in the recovery of major *S. fruticosa* phytochemicals.

Table 4. Quantitative information on major polyphenols considered to compare *S. fruticosa* extracts produced with 60% (*w*/*v*) glycerol/water (GL) and methyl *β*-cyclodextrin (m-*β*-CD).

Compound	Yield (mg g^{-1} dm) ± sd		
	m-*β*-CD	GL	% Difference
Chlorogenic acid	0.15 ± 0.02	0.24 ± 0.05	37.5
Luteolin 7-*O*-glucuronide	6.96 ± 1.12	5.51 ± 1.57	20.8
Rosmarinic acid	10.57 ± 1.37	10.63 ± 0.98	0.57
Sum	17.68	16.38	7.4

4. Conclusions

The approach attempted in this study aimed at (i) utilizing glycerol, a by-product of the biodiesel industry, as a green and non-volatile solvent, and (ii) integrating ultrasonication pretreatment as a step central to increasing the efficiency of the extraction methodology used. The combination of such a pretreatment with a hydroglycerolic solvent provided a high-efficiency extraction for *S. fruticosa* polyphenols. The kinetics showed that extraction at 50 °C may be the most favorable, and thus this methodology may also be energy-effective, a fact that significantly adds to the sustainable profile of the process. A prospect of this investigation would be future studies focusing on scale-up and application of hydroglycerolic extracts of *S. fruticosa* as effective food antioxidants/antimicrobials and/or cosmetic constituents. This would pave the way for the implementation of the process on an industrial scale.

Author Contributions: S.G. and A.H. carried out the experimentation and handled the raw data. D.P.M. conceived the idea, designed the experiment, performed statistics, handled the data, and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Batch Stirred-Tank Green Extraction of *Salvia fruticosa* Mill. Polyphenols Using Newly Designed Citrate-Based Deep Eutectic Solvents and Ultrasonication Pretreatment

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Abstract: A series of citrate salts were tested as hydrogen bond acceptors to synthesize deep eutectic solvents (DES) based on lactic acid and glycerol, used as hydrogen bond donors. The DES produced were then screened to identify the highest performing system for the effective extraction of polyphenolic phytochemicals from the medicinal plant *Salvia fruticosa* Mill. (Greek sage). The most efficacious DES was the one composed of lactic acid and sodium citrate dibasic, at a molar ratio of 15:1 (LA-SCDB15). Furthermore, for the first time there has been evidence concerning DES pH and extraction efficiency. Using this solvent, a batch, stirred-tank extraction process was developed, by employing ultrasonication pretreatment and response surface methodology. The optimal settings determined were stirring speed 900 rpm, proportion of DES/water 77% (*w*/*v*), and ultrasonication pretreatment time 15 min. By adjusting these optimal settings, the predicted maximum total polyphenol yield was calculated to be 79.93 ± 1.92 mg gallic acid equivalents g^{-1} dry mass. The examination of temperature effects demonstrated that the batch, stirred-tank extraction stage was very energy-efficient, with a barrier of 7.64 kJ mol^{-1}. Comparison of the extraction of *Salvia fruticosa* polyphenols with other green processes previously developed, illustrated the high extraction capacity of LA-SCDB15. The major polyphenols identified in the extracts produced under optimized settings were chlorogenic acid, luteolin 7-*O*-glucuronide and rosmarinic acid.

Keywords: antioxidants; deep eutectic solvents; extraction kinetics; polyphenols; *Salvia fruticosa*; ultrasonication

1. Introduction

Medicinal and aromatic plants (MAPs) are routinely used as food and folk remedies for centuries worldwide, and to-date substantial scientific evidence has accumulated to support their reputed nutritional and pharmacological properties. The knowledge derived by long-term traditional uses of MAPs has now been acknowledged as a sound basis to support health claims for numerous botanicals [1], and there has been a climbing interest for products originating from MAPs with a spectrum of bio-functionalities. Consumer trends for natural commodities with health-promoting activities has raised awareness and high demand for botanical-based supplements, and ignited a large

development of novel products, thus enabling the launch of a variety of functional ingredients [2] and cosmetic additives [3].

Currently, there is a great interest for the development and implementation of cutting-edge sustainable extraction methods for polyphenols from medicinal plants. In this direction, numerous green and low-cost approaches have gained acceptance as being more efficient and precise than traditional ones [4,5]. In compliance with green chemistry principles, a crucial concern towards establishing eco-friendly extraction processes is the replacement of conventional petroleum-based volatile solvents with bio-based alternatives. In this line, the utilization of a benign, eco-friendly solvent is of prime importance to the sustainable profile of an extraction process. Such a solvent should be non-toxic, it should have satisfactory extraction efficiency, it should be inexpensive and readily available, and it should originate from recyclable materials, such as waste biomass [6,7].

Deep eutectic solvents (DES) are innovative liquids, composed of low-cost, non-toxic and recyclable materials, which can be naturally occurring compounds (e.g., organic acids and salts, polyols, sugars, etc.). DES are usually composed of a substance functioning as hydrogen bond donor (HBD) (e.g., glycerol, organic acids) and another one as hydrogen bond acceptor (HBA) (e.g., choline chloride, amino acids), and their synthesis is straightforward and benign. DES possess features such as absence of flammability water (im)miscibility and low vapor pressure, and these attributes make DES suitable solvents for a spectrum of sustainable applications, such as extraction, synthesis, etc. [8]. To-date, by virtue of their unique properties, the use of DES for natural product extraction has been rapidly expanding, and there has been a bewildering number of substances used for DES synthesis.

The family of Lamiaceae is widespread and embraces 220 genera and 4000 species occurring around the globe. The chemistry of Lamiaceae species is exceptionally wide and versatile, as it concerns chemical constituents such as terpenes (diterpenes and triterpenes) and polyphenols, two major and multitudinous groups of biologically active compounds. *Salvia* L. is the largest genus of the Lamiaceae, represented by over than 1000 species [9,10]. *Salvia fruticosa* (syn. *S. triloba*), is known as Greek or Cretan sage, and it is a Lamiaceae species occurring in several parts of the East Mediterranean. A number of biological properties have been ascribed to this medicinal plant, which are mainly attributed to its polyphenolic load and composition [11–13]. However, up to now the development of green extraction processes for the production of polyphenol-containing bioactive extracts from *S. fruticosa* is extremely limited. Given the current expanding interest by several cosmetics and food supplement industries in Greece for this particular botanical, the current study had as objective the establishment of a batch stirred-tank green extraction methodology, by blending ultrasonication pretreatment and a highly efficacious DES, selected out of a thorough screening.

2. Materials and Methods

2.1. Chemicals

Chromatography solvents were HPLC grade. L-lactic acid (80%) was obtained from Fisher Scientific (Loughborough, UK). Sodium carbonate, sodium citrate dibasic sesquihydrate (>99%), sodium citrate monobasic (99%), sodium acetate trihydrate, ascorbic acid, rosmarinic acid, luteolin 7-*O*-glucoside, chlorogenic acid and 2,2-diphenylpicrylhydrazyl (DPPH) were from Sigma-Aldrich (Darmstadt, Germany). Sodium citrate tribasic dihydrate (>99%), Folin–Ciacalteu reagent, glycerol (99%) and citric acid and were from Merck (Darmstadt, Germany). Methanol and ethanol were from Honeywell/Riedel-de Haen (Seelze, Germany). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) and iron chloride hexahydrate were from Honeywell/Fluka (Steinheim, Germany).

2.2. Plant Material

Details regarding plant material source and handling have been described elsewhere [14]. In short, dry and powdered *S. fruticosa*, with mean particle size of 1.28 mm, was used in all experiments.

The material was from the area of Chania (Crete, southern Greece) and it composed of the aerial parts of the plant.

2.3. Preparation of the DES

To protocol followed for DES synthesis was based on a previously reported one [15]. Precise mass of HBD and HBA were mixed at various molar proportions, and the mixtures were heated at 70 °C, under continuous stirring at 500 rpm, until the formation of perfectly transparent liquids. This process usually required 60 min, depending on HBD/HBA combination and molar ratio. All DES produced were stored in glass screw-cap vials, at ambient temperature, in the dark, and they were periodically inspected for appearance of crystals over 5 weeks.

2.4. Ultrasonication Pretreatment

Ultrasonication of samples was applied prior to batch stirred-tank extraction, using an ultrasonication bath (Sonorex Bandeline, Berlin, Germany). The ultrsonication was carried out at ambient temperature, with the following settings: frequency, 100 Hz; power, 120 W; acoustic energy density, 120 W L^{-1}.

2.5. Batch Stirred-Tank Extraction Process

For the screening process, all DES were tested as 70% (*w*/*v*) aqueous mixtures. Control solvents were 60% (*v*/*v*) ethanol, 60% (*v*/*v*) methanol and deionized water [14,16]. Extractions were accomplished in a 20-mL glass vial, using 15 mL of each solvent and 0.375 g of plant material, for 150 min. Continuous stirring at 500 rpm and regulation of temperature at 50 °C were provided by a stirring hot plate (VELP Scientifica, San Francisco, CA, USA). After the extraction, extracts were centrifuged at 10,000× *g* for 10 min, to obtain a clear supernatant used for all determinations.

2.6. Design of Experiment and Response Surface Methodology

Response surface methodology was implemented through a Box–Behnken design with three central points, to assess the effect of selected process variables on the total polyphenol yield (Y_{TP}, mg GAE g^{-1} dm). The variables considered were the stirring speed (S_S, rpm), the DES/water proportion (C_{DES}, % *w*/*w*) and the ultrasonication pretreatment time (t_{US}, min), which were assigned as X_1, X_2 and X_3, respectively. Codification of the variable levels (Table 1) was done as described in detail elsewhere [16]. Model fitting to the experimental data was evaluated by performing ANOVA and lack-of-fit analysis, and non-significant dependent terms were excluded from the model (mathematical equation).

Table 1. Actual and coded levels of the independent variables selected to set up the experimental design.

Independent Variables	Code Units	Coded Variable Level		
		−1	**0**	**1**
S_S (rpm)	X_1	300	600	900
C_{DES} (%, *w*/*w*)	X_2	55	70	85
t_{US} (min)	X_3	5	10	15

2.7. Extraction Kinetics

The kinetic model employed has been previously reported [17]. The model is described by the following equation:

$$Y_{TP(t)} = \frac{Y_{TP(s)}\, t}{t_{0.5} + t} \quad (1)$$

$Y_{TP(t)}$ and $Y_{TP(s)}$ correspond to the yield in total polyphenols at any time, t, and at saturation (equilibrium). The term $t_{0.5}$ corresponds to the time at which $Y_{TP(t)} = \frac{Y_{TP(s)}}{2}$. The initial extraction rate, h, and the second-order extraction rate, k, can be determined by the following equations:

$$h = \frac{Y_{TP(s)}}{t_{0.5}} \quad (2)$$

$$k = \frac{1}{Y_{TP(s)}\ t_{0.5}} \quad (3)$$

The influence of temperature on k was portrayed by non-linear regression between k and T. Effective description of this correlation could be given by an exponential model, as previously proposed [18]:

$$k = k_0 + ae^{-bT} \quad (4)$$

Term k corresponds to the second-order extraction rate and k_0 is a pre-exponential factor. Where a and b are fitting parameters. Estimation of the activation energy (E_a) of the process was calculated as follows [19]:

$$ln\left(\frac{k}{k_{ref}}\right) = \left(-\frac{E_a}{R}\right)\left(\frac{1}{T} - \frac{1}{T_{ref}}\right) \quad (5)$$

T_{ref} and T represent a reference temperature (K) and a temperature at which kinetics was traced, k_{ref} and k correspond to the second-order extraction rate constants, R is the universal gas constant (8.314 J K^{-1} mol^{-1}) and E_a the activation energy (J mol^{-1}).

2.8. Determinations

Total polyphenol concentration was determined with the Folin–Ciocalteu methodology and yield in total polyphenols was expressed as mg gallic acid equivalents (GAE) per g dry mass [20]. Total flavonoid determination was carried out with the aluminum chloride reagent and results were expressed as mg rutin equivalents (RtE) per g dry mass [21]. The antiradical activity (A_{AR}) was estimated with a stoichiometric methodology [16], using DPPH as the radical probe. The ferric-reducing power was measured with a modified FRAP assay and expressed as µmol ascorbic acid equivalents (AAE) per g dry mass [16].

2.9. Chromatographic Analyses

The equipment used was a FinniganMAT P4000 pump, coupled with a UV6000LP diode array detector (Thermo Scientific, Waltham, MA, USA), and a TSQ Quantum Access LC/MS/MS, with a surveyor pump (Thermo Scientific, Walltham, MA, USA), interfaced by XCalibur 2.1, TSQ 2.1 software. Chromatographic analyses were performed on a Superspher RP-18 column, 125 mm × 2 mm, 4 µm, at 40 °C, with a 10 µL injection loop. The eluents were (A) 2.5% acetic acid and (B) methanol. The flow rate was 0.3 mL min^{-1}, and the elution program used was: 0 min, 100% A; 22 min, 65% A; 32 min, 65% A; 60 min, 0% A; 65 min, 0% A. Mass spectra acquisition was performed with negative ionization, capillary temperature 300 °C, sheath gas pressure 30 mTorr, auxiliary gas pressure 15 mTorr, and collision pressure at 1.5 mTorr; quantification was done with external standards, using a rosmarinic acid (50–3000 µg L^{-1}, R^2 = 0.9985) and a luteolin 7-*O*-glucoside (5–1500 µg L^{-1}, R^2 = 0.9982) calibration curve. Standards were prepared in HPLC grade methanol and stored at −17 °C.

2.10. Statistics

Design of experiment, statistics associated with response surface methodology (ANOVA, lack-of-fit) and distribution analysis was performed with JMP™ Pro 13 (SAS, Cary, NC, USA). Linear regressions, non-linear regressions and kinetics model fitting were performed with SigmaPlot™

12.5 (Systat Software Inc., San Jose, CA, USA). Extraction experiments were carried out at least twice and all determinations in triplicate. Values given are averages ± standard deviation.

3. Results and Discussion

3.1. Screening of DES for Extraction Efficiency

The evidence emerged from a previous investigation suggested that citrate salts may form DES with increased extraction efficiency [15]. Thus, the generation of a series of DES was systematically approached, by selecting two widely used HBDs, glycerol (GL) and L-lactic acid (LA), and citrate salts as the HBAs (Figure 1). The salts tested were sodium citrate monobasic (SCMB), sodium citrate dibasic (SCDB) and sodium citrate tribasic (SCTB). However, as attempts to synthesize DES with either GL or LA and SCMB did not meet with success, even when HBD:HBA molar ratio ($R_{mol}^{D/A}$) was 15. Thus, SCMB was not further considered. With regard to SCDB, it formed stable DES (no crystallization) with GL and LA at $R_{mol}^{D/A} \geq 9$; therefore, a series of GL-SCDB and LA-SCDB DES were synthesized with $R_{mol}^{D/A}$ varying from 9 to 15. On the other hand, SCTB formed stable DES with GL only at a $R_{mol}^{D/A}$ of 15. By contrast, stable DES with LA and SCTB were formed at $R_{mol}^{D/A} \geq 7$. Thus, LA-SCTB DES were tested within a $R_{mol}^{D/A}$ range of 7 to 15.

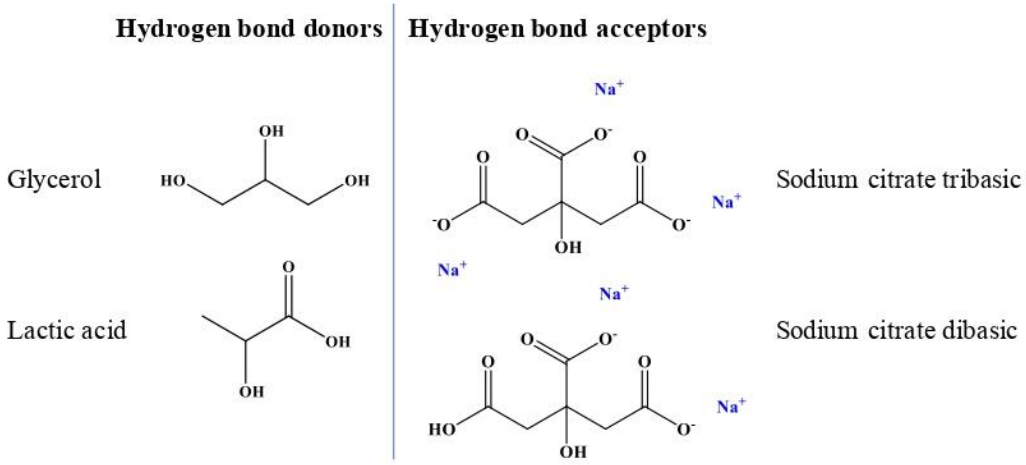


Figure 1. Hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) tested in the current investigation.

In total, 14 DES were tested covering a wide pH range, from 2.86 (LA-SCDB15) to 7.50 (GL-SATB15). The extraction efficiency of the DES synthesized was compared to other green solvents, including water and 60% (*v*/*v*) ethanol, but also 60% (*v*/*v*) methanol, which is a commonly used solvent for polyphenol extraction (Figure 2). The highest Y_{TP} was found for the extraction with 60% (*v*/*v*) methanol (67.86 ± 1.70 mg GAE g^{-1} dm), followed by 60% (*v*/*v*) ethanol (55.64 ± 1.39 mg GAE g^{-1} dm). Regarding the DES, the LA-SCDB with $R_{mol}^{D/A} = 15$, termed as LA-SCDB15, gave a Y_{TP} of 52.31 ± 1.31 mg GAE g^{-1} dm and it was the most efficient one ($p_{hp} < 0.05$), as opposed to GL-SATB15, which was the least efficient (31.49 ± 0.63 mg GAE g^{-1} dm). Because it was observed that these extreme Y_{TP} values coincided with the corresponding extreme pH values, concerns were raised as to what extent the pH of a DES could affect polyphenol extractability.

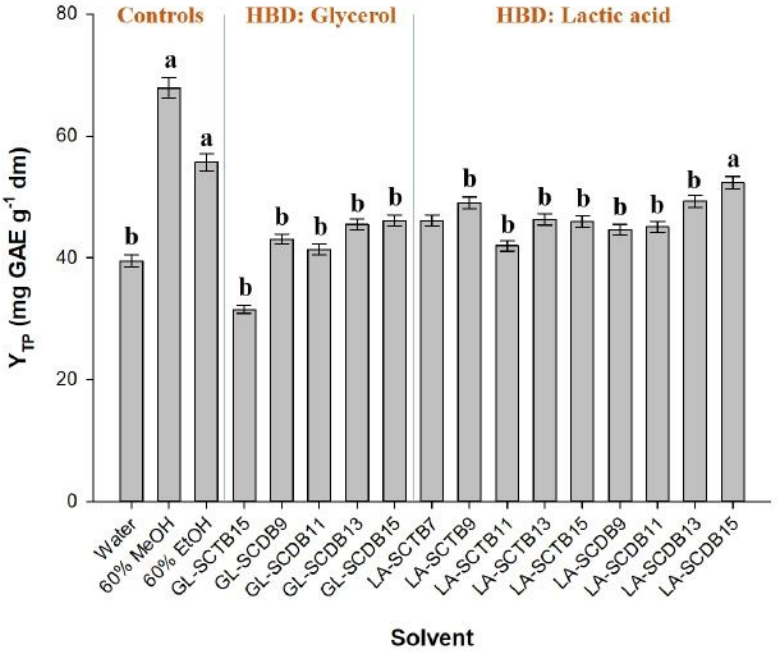


Figure 2. Graph showing the results of screening of the deep eutectic solvents (DES) tested. Extractions were accomplished at 50 °C, for 150 min, under continuous stirring at 500 rpm. All DES were tested as 70% (*w*/*v*) aqueous mixtures. Values designated with different letters are statistically different ($p < 0.05$).

To obtain evidence for such an effect, the pH values of all DES tested were plotted against Y_{TP} (Figure 3). The linear regression gave $R^2 = 0.67$ ($p = 0.0003$), revealing a trend that should not be overlooked, which evidenced higher extraction efficiency for DES with lower pH. Although previous studies on polyphenol extraction with DES stressed emphatically the importance of $R^{D/A}_{mol}$ on the extraction yield [16,21–24], a correlation of yield with pH is heretofore unreported.

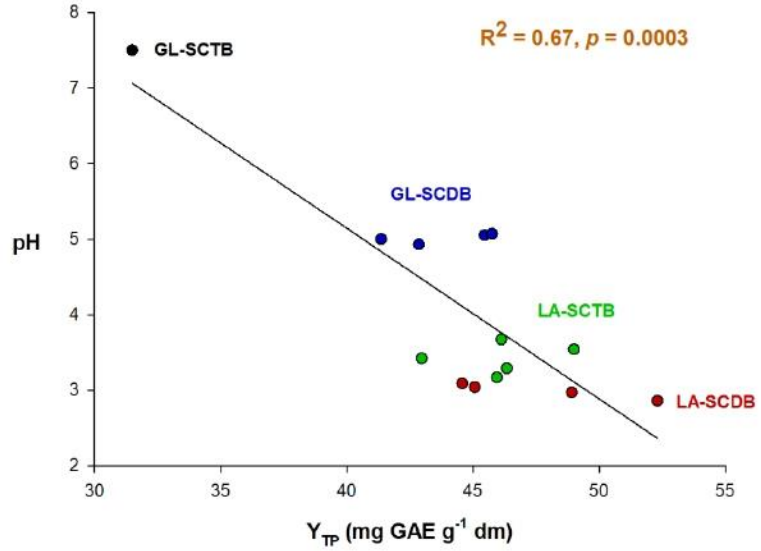


Figure 3. Linear regression between the pH of the DES tested and Y_{TP}. All DES were tested as 70% (*w*/*v*) aqueous mixtures.

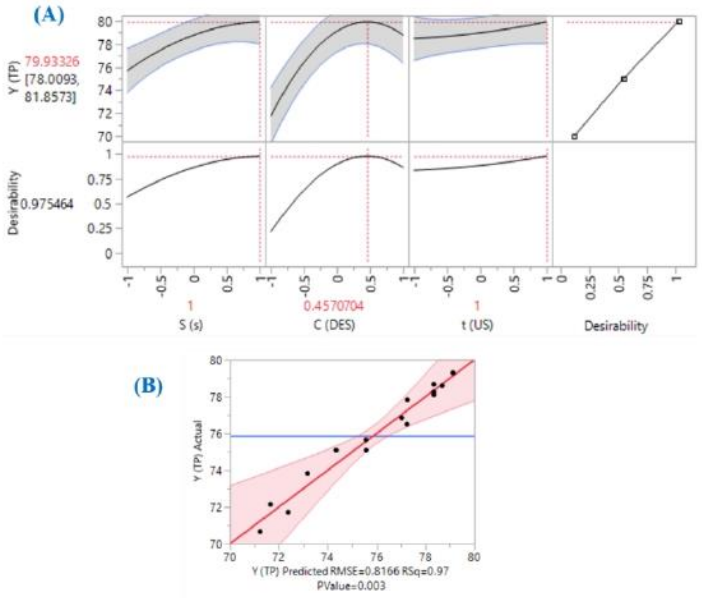
As also reported for classical solvents, pH is critical for DES extraction efficiency. Earlier investigations with conventional volatile solvents addressed the role of pH on polyphenol extractability, demonstrating that higher total polyphenol yield from olive leaves could be achieved at pH 2 [25]. Results from following studies on onion solid wastes were in the same line, indicating pH 2 as optimal

to maximize polyphenol extraction [26]. Furthermore, examinations on grape stem [27] and grape seed [28] polyphenol extraction showed that in most cases higher yields in total polyphenols, total flavanols and proanthocyanidins were favored at pH < 3.5. For other plant tissues, such as *Solanum melongena*, effective extraction was accomplished with 70% ethanol adjusted at pH 3 [29]. Such a phenomenon was ascribed to the protective effect of low pH on polyphenol against polyphenol oxidation because polyphenol oxidizability is higher at neutral or alkaline environment, due to phenolic hydroxyl dissociation. Likewise, it could be argued that acidic DES might act protectively with regard to polyphenol oxidation, and this would be likely to contribute to achieving higher Y_{TP}.

3.2. Extraction Process Optimization

Since LA-SCDB15 provided significantly higher Y_{TP} compared to all other DES tested, this solvent was chosen to further optimize the extraction process. To this end, three process variables that can critically affect polyphenol extraction [16,21,24], namely the S_S, the C_{DES} and the t_{US}, were included in the experimental design. The design deployed aimed at assessing the effect of the process variables and identifying any synergistic functions between them. The evaluation of model fitting and validity was based on the ANOVA and lack-of-fit test (Figure 4), taking into account the proximity of measured and predicted values (Table 2). The mathematical model derived after omitting non-significant terms, was as follows:

$$Y_{TP} = 78.34 + 1.31X_1 + 1.70X_2 + 1.09X_2X_3 - 3.86X_2^2 \ (R^2 = 0.97, p = 0.003) \quad (6)$$



Lack Of Fit

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	3	3.1640250	1.05468	12.4177
Pure Error	2	0.1698667	0.08493	Prob > F
Total Error	5	3.3338917		0.0754
				Max RSq

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	78.343333	0.471444	166.18	<.0001*
S (s)	1.30625	0.288699	4.52	0.0063*
C (DES)	1.70375	0.288699	5.90	0.0020*
t (US)	-0.255	0.288699	-0.88	0.4175
S (s)*C (DES)	0.7375	0.408282	1.81	0.1307
S (s)*t (US)	0.475	0.408282	1.16	0.2972
C (DES)*t (US)	1.09	0.408282	2.67	0.0444*
S (s)*S (s)	-0.967917	0.424954	-2.28	0.0717
C (DES)*C (DES)	-3.862917	0.424954	-9.09	0.0003*
t (US)*t (US)	0.2245833	0.424954	0.53	0.6198

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
S (s)	1	1	13.650312	20.4720	0.0063*
C (DES)	1	1	23.222112	34.8273	0.0020*
t (US)	1	1	0.520200	0.7802	0.4175
S (s)*C (DES)	1	1	2.175625	3.2629	0.1307
S (s)*t (US)	1	1	0.902500	1.3535	0.2972
C (DES)*t (US)	1	1	4.752400	7.1274	0.0444*
S (s)*S (s)	1	1	3.459185	5.1879	0.0717
C (DES)*C (DES)	1	1	55.097078	82.6318	0.0003*
t (US)*t (US)	1	1	0.186231	0.2793	0.6198

Figure 4. Statistics associated with model fitting, performed by implementing response surface methodology. (**A**), Desirability function; and (**B**), actual-by-predicted plot. Inset tables (lack-of-fit, parameter estimates and effect test) illustrate the effect of independent (process) variables on the response. Asterisk (*) on values in the "parameter estimates" and "test effects" inset tables signify statistically significant values (at least at a 95% significance level).

Table 2. Analytical presentation of the design of experiment (design points), including predicted and measured values of the response.

Design Point	Independent Variables			Response	
	X_1 (S_S, rpm)	X_2 (C_{DES}, % *w*/*v*)	X_3 (t_{US}, min)	Y_{TP} (mg GAE g^{-1} dm)	
				Measured	Predicted
1	−1 (300)	−1 (55)	0 (10)	70.67	71.24
2	−1 (300)	1 (85)	0 (10)	73.83	73.17
3	1 (900)	−1 (55)	0 (10)	71.72	72.38
4	1 (900)	1 (85)	0 (10)	77.83	77.26
5	0 (600)	−1 (55)	−1 (5)	75.09	74.35
6	0 (600)	−1 (55)	1 (15)	72.14	71.66
7	0 (600)	1 (85)	−1 (5)	75.09	75.57
8	0 (600)	1 (85)	1 (15)	76.50	77.24
9	−1 (300)	0 (70)	−1 (5)	76.85	77.02
10	1 (900)	0 (70)	−1 (5)	78.60	78.69
11	−1 (300)	0 (70)	1 (15)	75.65	75.56
12	1 (900)	0 (70)	1 (15)	79.30	79.13
13	0 (600)	0 (70)	0 (10)	78.67	78.34
14	0 (600)	0 (70)	0 (10)	78.25	78.34
15	0 (600)	0 (70)	0 (10)	78.11	78.34

The square correlation coefficient (R^2) was a good indicator of the total variability around the mean provided by the Equation (6). Assuming a confidence interval of 95% and considering the R^2 the *p* value for lack-of-fit (Figure 4), it could be supported that the mathematical model displayed very satisfactory adjustment to the experimental data. The 3D graphs that represent a visualization of the model, can portray at-a-glance the effect of the process variables on the response (Y_{TP}) (Figure 5). The desirability function (Figure 4) provided the theoretical optimized values for each of the variables considered, which were S_S = 900 rpm, C_{DES} = 77% (*w*/*v*), and t_{US} = 15 min. By adjusting these optimal settings, the predicted maximum response was calculated to be 79.93 ± 1.92 mg GAE g^{-1} dm. To ascertain the validity of the model, three individual extracts were performed using the optimized values and the outcome was 78.39 ± 2.96 mg GAE g^{-1} dm, illustrating the accuracy of response prediction.

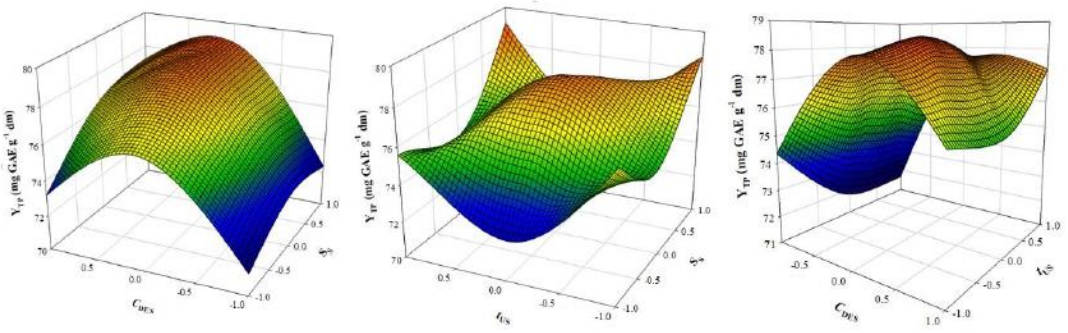


Figure 5. The effect of independent variables on the response (Y_{TP}), illustrated as three-dimensional plots.

The ANOVA results indicated that X_3 (t_{US}) was not statistically significant, as opposed to its cross term with X_2 (C_{DES}) (Figure 4). This finding pointed to a combined effect of these two variables and evidenced that the efficiency of the ultrasonication pretreatment might be dependent on the proportion of DES/water. On the other hand, X_1 (S_S) had a clear positive effect on Y_{TP}, which showed that increased speed of agitation favored polyphenol extraction yield. Recent studies on the effect of S_S on Y_{TP} gave contradictory results, suggesting that the influence exerted by S_S might not follow a specific pattern.

In certain cases, such as polyphenol extraction from hop [16], saffron processing wastes [22] and onion solid wastes [30], relatively high S_S (>650 rpm) were demonstrated to provide Y_{TP} maximization.

To the contrary, the requirement in S_S for optimum extraction of olive leaf polyphenol was shown to be either low (300 rpm) [31] or moderate (500 rpm) [21]. In another study on the extraction of polyphenols from *M. oleifera* leaves, the optimum S_S was determined to be 800 rpm, but when ultrasonication was integrated as pretreatment, the optimum S_S was 200 rpm [23]. In general, S_S is considered to play important role in solid–liquid extraction, and its careful adjustment may end up in significantly higher yields [32,33]. It has been supported that a sufficient level of S_S results in turbulence in the extraction tank, which is appropriate to boost mass transfer rate, and increases in S_S have been correlated to higher polyphenol diffusivity [33].

C_{DES} had also a significant positive effect on Y_{TP}, and the optimum value estimated was 77% (*w*/*v*). This level lies between 75 and 80% (*w*/*v*) found for polyphenol extraction with DES from *M. oleifera* leaves [23,34], and 78 and 80% (*w*/*v*) from olive leaves [21,35]. Other investigations reported 80% (*w*/*w*) for tartary buckwheat hull [36], 80% (*w*/*w*) for sea buckthorn leaves [37], 74% (*w*/*w*) for *Cymbidium kanran* [38] and 76.2% (*w*/*w*) for grape skin [39]. In all these optimization studies, the appropriate adjustment of water amount was shown to be critical for the extraction efficiency, because the DES/water proportion regulates features such as viscosity and polarity [40], which profoundly affect solute solubility, hence extraction performance. Such hypothesis has been well exemplified by a recent examination, which demonstrated that the higher the lipophilicity of the HBA in a DES, the higher the water amount required to achieve polyphenol extraction maximization from *O. dictamnus* [41].

3.3. Extraction Kinetics—Temperature Effects

Previous studies on the extraction of polyphenols from *S. fruticosa* using methyl β-cyclodextrin (m-β-CD) showed that extracts with increased polyphenol concentration and improved antioxidant characteristics could be obtained at 80 °C [14]. However, a following investigation with a 60% (*w*/*v*) hydroglycerolic mixture demonstrated that Y_{TP} displayed a gradual decrease when extraction temperature varied from 50 to 80 °C [42], although differences were non-significant ($p < 0.05$). Therefore, to obtain a reliable picture of the effect of temperature, extraction kinetics was traced within the range of 40 to 80 °C (Figure 6), under optimized conditions, that is, S_S = 900 rpm, C_{DES} = 77% (*w*/*v*), and t_{US} = 15 min.

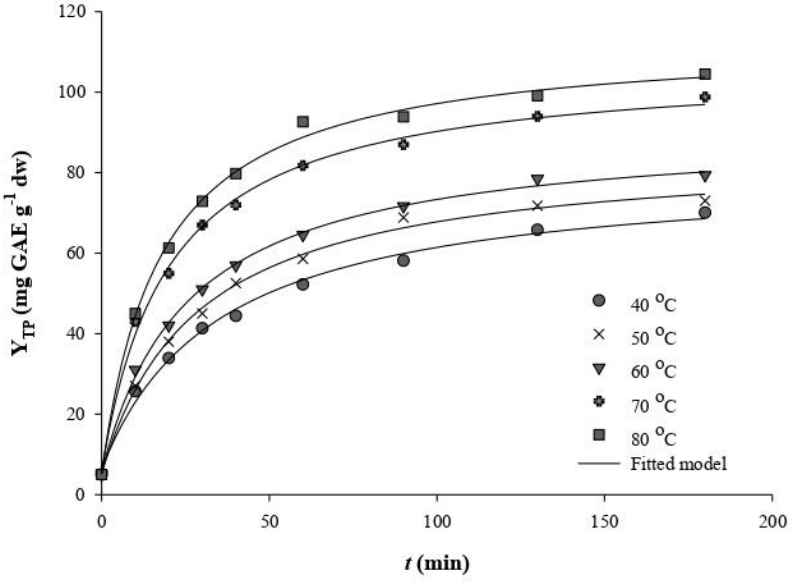


Figure 6. Kinetics of polyphenol extraction from *S. fruticosa*, traced under optimized conditions (S_S = 900 rpm, C_{DES} = 77% (*w*/*v*), and t_{US} = 15 min).

Y_{TP} exhibited an increasing trend, and at 80 °C the $Y_{TP(s)}$ determined was 113.39 mg GAE g^{-1} dm (Table 3). Likewise, the initial extraction rate, h, increased from 2.314 mg g^{-1} min^{-1} at 40 °C to 6.439 mg g^{-1} min^{-1} at 80 °C, and $t_{0.5}$ showed a declining tendency over this range, which manifested acceleration of the extraction. In a similar manner, the second-order extraction rate, k, increased from 0.356×10^{-3} g mg^{-1} min^{-1} at 40 °C to 0.501×10^{-3} g mg^{-1} min^{-1} at 80 °C, and k values correlated well with T ($R^2 = 0.96$, $p = 0.0413$), using the exponential model described by the Equation (4) (Figure 7). Comparison with extraction using hydroglycerolic solvent [42] showed that the fitting parameter b (Equation (4)), which is a measure of the sensitivity of k with regard to T changes, was 0.0136 for the extraction with LA-SCDB15 and 0.0765 for the extraction with hydroglycerolic solvent. This finding suggested that the extraction with hydroglycerolic solvent was more energy-demanding.

Table 3. Illustration of the data derived by implementing kinetics to assess the effect of T on the extraction of *S. fruticosa* polyphenols, under optimized conditions.

T (°C)	Kinetic Parameters				
	k ($\times 10^{-3}$) (g mg^{-1} min^{-1})	H (mg g^{-1} min^{-1})	$Y_{TP(s)}$ (mg GAE g^{-1})	$t_{0.5}$ (min)	E_a (kJ mol^{-1})
40	0.356	2.314	80.64 [a]	34.85	
50	0.407	2.994	85.73 [a]	28.63	
60	0.424	3.508	90.95 [a]	25.93	7.64
70	0.471	5.388	107.01 [a]	19.86	
80	0.501	6.439	113.39 [b]	17.61	

Values with different letters within the same column are statistically different ($p < 0.05$).

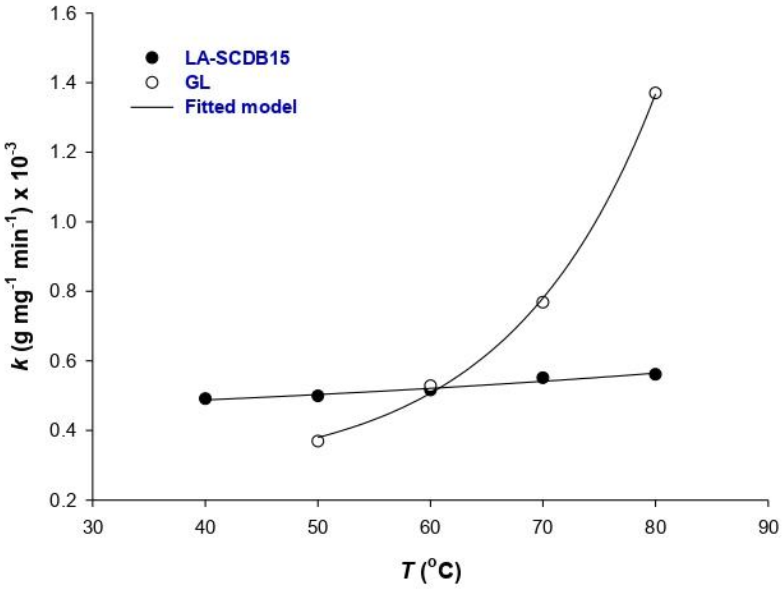


Figure 7. Non-linear regression between second-order extraction rate values, k, and T. Data concerning the extraction with 60% glycerol/water (GL) were obtained from Grigorakis et al., 2020.

To corroborate this hypothesis, the activation energy (E_a) of the process was estimated using the Equation (5). The barrier level of 7.64 kJ mol^{-1} found was significantly lower than 47.67 kJ mol^{-1} determined for the extraction with 60% (w/v) glycerol [42], thus affirming the higher efficiency of the extraction with LA-SCDB15. At this point, it should be stressed that in both cases stirred-tank extraction took place after ultrasonication pretreatment. This pretreatment stage resulted in washing out the most readily extracted compounds, a phenomenon also observed in other cases [16,21] and therefore the E_a determined corresponded to the extraction of the remaining solute, whose dissolution and entrainment into the liquid phase is governed by internal diffusion. The fact that the stirred-tank stage was far less energy-demanding using LA-SCDB15 than 60% (w/v) glycerol, evidenced that this

solvent might provide higher polyphenol solubility or that it might penetrate easier into the solid particles, or both.

3.4. Polyphenolic Profile and Antioxidant Activity—Comparative Assessment

To further bring out the efficiency of LA-SCDB15, the characteristics of an extract obtained under optimized conditions were compared to those from two preexisting green extraction methods, one performed with methyl β-cyclodextrin (m-β-CD) [14] and one with 60% (*w*/*v*) glycerol/water mixture (GL) [42], but also 60% (*v*/*v*) aqueous ethanol and 60% (*v*/*v*) aqueous methanol (Table 4).

Table 4. Comparative assessment of *S. fruticosa* extracts produced with LA-SCDB15 and other green solvents. Values given represent means ± standard deviation.

Extract	Y_{TP} (mg GAE g^{-1} dm)	A_{AR} (μmol DPPH g^{-1} dm)	P_R (μmol AAE g^{-1} dm)
Water	63.72 ± 0.96	613.07 ± 12.26 [a]	529.14 ± 7.94 [a]
60% MeOH	84.71 ± 1.27	828.54 ± 8.29 [b]	703.98 ± 10.56 [b]
60% EtOH	87.66 ± 1.31	820.45 ± 16.41 [b]	684.20 ± 10.26 [b]
m-β-CD	85.54 ± 1.28	820.93 ± 16.42 [b]	590.66 ± 14.77 [b]
GL	87.26 ± 1.31	817.58 ± 8.18 [b]	709.12 ± 17.73 [b]
LA-SCDB15	98.05 ± 1.47 [a]	751.74 ± 7.52 [b]	521.85 ± 7.83 [a]

Values with different letters within the same column are statistically different ($p < 0.05$).

The LA-SCDB15 extract was found to have significantly higher Y_{TP}, which demonstrated its high extraction capacity. Furthermore, the extract displayed A_{AR} comparable to the other extracts, except for water extract, where the A_{AR} was significantly weaker. On the other hand, both LA-SCDB15 and water extracts exhibited significantly lower P_R.

Three major *S. fruticosa* polyphenols occurring in LA-SCDB15 extracts were considered for quantification (Figure 8), and the results were compared to GL and m-β-CD. As can be seen in Table 5, extraction with LA-SCDB15 afforded by 31.8% higher yield in chlorogenic acid compared to m-β-CD, but by 8.3% less so compared to GL. On the other hand, the yield attained with LA-SCDB15 for luteolin 7-*O*-glucuronide was by only 2.7% higher than that attained with m-β-CD, but by 23% higher than that achieved with GL. Likewise, extraction with LA-SCDB15 performed by 38 and 37.6% higher than that with m-β-CD and GL, respectively, with regard to rosmarinic acid recovery. Overall, the extraction with LA-SCDB15 was by 27.6 and 32.9% more efficient than the corresponding carried out with m-β-CD and GL.

Table 5. Extraction yield in principal polyphenolic phytochemicals of *S. fruticose*, using LA-SCDB15, methyl β-cyclodextrin (m-β-CD) and 60% (*w*/*v*) glycerol/water (GL). Values reported are means ± standard deviation.

Compound	Yield (mg g^{-1} dm) ± sd		
	m-β-CD	GL	LA-SCDB15
Chlorogenic acid	0.15 ± 0.02 [a]	0.24 ± 0.05 [b]	0.22 ± 0.00 [b]
Luteolin 7-*O*-glucuronide	6.96 ± 1.12 [a]	5.51 ± 1.57 [b]	7.15 ± 0.37 [a]
Rosmarinic acid	10.57 ± 1.37 [a]	10.63 ± 0.98 [a]	17.04 ± 0.15 [b]
Sum	17.68 [a]	16.38 [a]	24.41 [b]

Values with different letters within the same row are statistically different ($p < 0.05$).

The outcome presented in Tables 4 and 5 pointed out the higher efficiency of LA-SCDB15 and it was in line with earlier examinations, which demonstrated that polyphenol extraction with DES was more effective than those performed with common conventional solvents, such as aqueous methanol or ethanol [16,21,23,24]. At this point it should be stressed that the content of *S. fruticosa* in certain major polyphenolic phytochemicals depends to a large extent by the time of collection. For example, it has been illustrated that the content of rosmarinic acid, which is the main *S. fruticosa* polyphenol, may vary from 5.57 to as high as 45.06 mg g^{-1} dm, and that of chlorogenic acid from 0.46 to 1.82 mg g^{-1} dm [12].

Seasonal ranges between 4.73 and 6.29, and 0.042 and 0.15 mg g^{-1} dm, for rosmarinic and chlorogenic acid, respectively, have also been determined [43]. However, other authors reported seasonal variation of rosmarinic acid to be between 0.20–1.70 mg g^{-1} dm [44]. Levels of rosmarinic acid reported in Greek *S. fruticosa* specimens were 14.83 mg g^{-1} dm [45] and 27.8–76.6 mg g^{-1} dm [46].

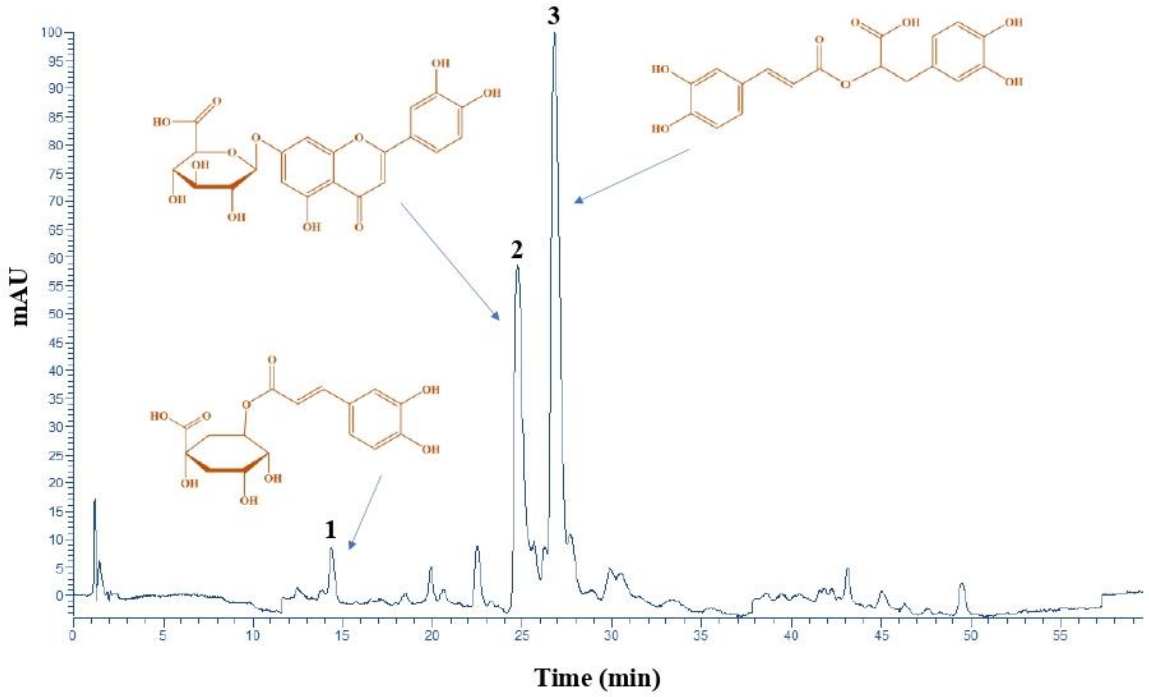


Figure 8. Chromatographic analysis of polyphenols in a *S. fruticosa* extract, produced under optimized conditions (S_S = 900 rpm, C_{DES} = 77% (*w*/*v*), and t_{US} = 15 min). The chromatogram was obtained at 330 nm. Peak assignment: 1, chlorogenic acid; 2, luteolin 7-*O*-glucuronide; 3, rosmarinic acid.

4. Conclusions

In the study presented herein, there has been a systematic approach to identify the most effective DES for the extraction of *S. fruticosa* polyphenols, by screening several citrate salts combined with two common HBDs, lactic acid and glycerol. The highest performing system was a DES composed of lactic acid and sodium citrate dibasic, at a molar ratio of 15:1, and for the first time, there has been evidence that the extraction performance of DES might depend on their pH. Optimization of the extraction and examination of the effect of temperature showed that blending ultrasonication pretreatment with optimized stirred-tank extraction may be a highly efficient green method to produce polyphenol-enriched extracts from *S. fruticosa*. This was also demonstrated by comparison with other pre-existing green extraction methodologies. The major polyphenolic phytochemicals identified in the extracts produced under optimized conditions were chlorogenic acid, luteolin 7-*O*-glucuronide and rosmarinic acid. The method developed is proposed as a green and efficacious methodology to recover bioactive polyphenols from the medicinal plant *S. fruticosa*. Testing of this solvent on several other matrices and comparison with other natural DES may reveal its full potential. Such a work is currently under progress.

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