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<sub>CD</sub>), pH, and liquid-to-solid ratio (R<sub>L/S</sub>), 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<sup>-1</sup> 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 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].

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, U.S.A.). 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<sub>CD</sub>), pH, and liquid-to-solid ratio (R<sub>L/S</sub>) 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<sub>TP</sub>) was the screening response and codification of the variables chosen (C<sub>CD</sub>, pH, R<sub>L/S</sub>) between −1 (lower limit) and 1 (upper limit), and was performed as follows:

\[
x_i = \frac{X_i - X_0}{\Delta X_i}, \quad i = 1, 2, 3
\]

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 \(\Delta X_i\) 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 R² 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<sub>TP</sub> and visualization of the independent variable effect on Y<sub>TP</sub> 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 |
|-----------------------|------------|----------------------|
| C<sub>D</sub> (% w/v)  | X<sub>1</sub> | -1 0.60 1.00 1.40    |
| R<sub>L/S</sub> (mL g<sup>-1</sup>) | X<sub>2</sub> | 20 60 100          |
| pH                   | X<sub>3</sub> | 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<sub>2</sub>CO<sub>3</sub> 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<sub>TP</sub>, mg L<sup>-1</sup>) was calculated from a calibration curve, constructed with gallic acid as standard. Total polyphenol yield (Y<sub>TP</sub>) was estimated by the following equation and expressed as mg gallic acid equivalents (GA<sub>E</sub>s) g<sup>-1</sup> dry mass (dm):

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

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<sub>AR</sub>) Measurement

For the determination A<sub>AR</sub>, 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<sub>515(i)</sub>). The mixture was allowed to react for 30 min and then recording of absorbance at 515 nm was repeated (A<sub>515(f)</sub>). A<sub>AR</sub> was calculated as follows:

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

where C<sub>DPPH</sub> is the DPPH concentration (μM) and C<sub>TP</sub> is the total polyphenol concentration (mg L<sup>-1</sup>) in the reaction mixture; A<sub>515(i)</sub> is the A<sub>515</sub> at t = 0 min and A<sub>515(f)</sub> the A<sub>515</sub> at t = 30 min; and Y<sub>TP</sub> is the total polyphenol yield (mg g<sup>-1</sup>) of the extract. A<sub>AR</sub> was expressed as μmol DPPH g<sup>-1</sup> dm.

2.7. Ferric-Reducing Power (PR) Determination

PR 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<sub>3</sub> (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. PR was determined from an ascorbic acid calibration curve (50–300 μM) and given as μM ascorbic acid equivalents (AA<sub>E</sub>s) g<sup>-1</sup> 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, U.S.A.). 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<sup>-1</sup>.

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 °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, R² = 0.9982) and a rosmarinic acid (50–3000 μg L⁻¹, R² = 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, RL/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 (R²) of the models, which are indicators of the total variability around the mean determined by the model. Because all total R² 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 RL/S or pH was significant, showing that combined effects did not occur. Contrary to those, for the extractions performed with any CD, both RL/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 RL/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.
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<sub>CD</sub>            | 0.518449       | 1.71    | 0.1471          | 239.91451      | 2.9386  |
| R<sub>LS</sub>            | 0.518449       | 10.56   | 0.0001 *        | 252.90005      | 111.5718|
| pH                        | 0.518449       | -10.84  | 0.0001 *        | 12.14523       | 117.6107|
| C<sub>CD</sub> R<sub>LS</sub> | 0.733198     | -2.38   | 0.0634          | 0.11560        | 5.6481  |
| C<sub>CD</sub> pH         | 0.733198       | -0.23   | 0.8258          | 0.65610        | 0.0538  |
| R<sub>LS</sub> pH         | 0.733198       | -0.55   | 0.6045          | 1.93408        | 0.3051  |
| C<sub>CD</sub> C<sub>CD</sub> | 0.763136    | -0.95   | 0.3865          | 53.14168       | 0.8994  |
| R<sub>LS</sub> R<sub>LS</sub> | 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<sub>CD</sub>            | 0.644945       | 0.75    | 0.4859          | 298.77901      | 0.5655  |
| R<sub>LS</sub>            | 0.644945       | 9.48    | 0.0002 *        | 130.81531      | 89.7874 |
| pH                        | 0.644945       | -6.27   | 0.0015 *        | 3.18623        | 39.3119 |
| C<sub>CD</sub> R<sub>LS</sub> | 0.912089       | 0.98    | 0.3728          | 6.94323        | 0.9575  |
| C<sub>CD</sub> pH         | 0.912089       | 1.44    | 0.2082          | 22.94410       | 2.0865  |
| R<sub>LS</sub> pH         | 0.912089       | -2.63   | 0.0468 *        | 1.93186        | 6.8950  |
| C<sub>CD</sub> C<sub>CD</sub> | 0.949333       | 0.76    | 0.4805          | 9.20776        | 0.5806  |
| R<sub>LS</sub> R<sub>LS</sub> | 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<sub>CD</sub>            | 0.273577       | 7.60    | 0.0047 *        | 195.89960      | 57.7357 |
| R<sub>LS</sub>            | 0.335062       | 18.09   | 0.0004 *        | 193.40255      | 327.1775|
| pH                        | 0.335062       | -17.97  | 0.0004 *        | 0.18923        | 323.0072|
| C<sub>CD</sub> R<sub>LS</sub> | 0.386897       | -0.56   | 0.6133          | 3.27610        | 0.3160  |
| C<sub>CD</sub> pH         | 0.386897       | 2.34    | 0.1013          | 17.09663       | 5.4715  |
| R<sub>LS</sub> pH         | 0.547155       | 5.34    | 0.0128 *        | 6.91763        | 28.5536 |
| C<sub>CD</sub> C<sub>CD</sub> | 0.47385        | 3.40    | 0.0425 *        | 20.80413       | 11.5533 |
| R<sub>LS</sub> R<sub>LS</sub> | 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 YTP values for the extractions carried out with each of the CDs used. GAE, gallic acid equivalent.

| Design Point | Independent Variables | Response (YTP, mg GAE g⁻¹ dw) |  |  |  |
|--------------|-----------------------|--------------------------------|---|---|---|
|              | C_CD (X₁) | R_L/S (X₂) | pH (X₃) | 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 4. Mathematical models and associated statistics derived from the experimental design.

| Cyclodextrin | 2nd Order Polynomial Equations | R²  | p     |
|--------------|-------------------------------|-----|-------|
| β-CD         | 43.60 + 5.48X₁ - 5.63X₂ - 3.79X₃ - 4.74X₁X₂ | 0.98 | 0.0006 |
| HP-β-CD      | 46.36 + 6.11X₁ - 4.04X₂ - 2.39X₁X₃ - 4.66X₂X₃ | 0.97 | 0.0025 |
| m-β-CD       | 47.75 + 2.08X₁ + 6.06X₂ - 6.02X₃ + 2.92X₁X₃ - 2.79X₂X₃ - 5.48X₃ | 1.00 | 0.0024 |
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.

The desirability function (Figure 3) enabled the estimation of the optimal predicted response for each CD tested (Table 5). The YTP 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.
For all CDs, optimum RL/S varied closely within 93–100 mL g⁻¹ (Table 5). This outcome showed that the influence exerted by RL/S on the extraction performance was not significantly affected by the structure of CD. The magnitude of RL/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 RL/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 RL/S optima between 81 [16] and 100 mL g⁻¹ [17–19]. Considering that the average RL/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.

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 YTP (± sd) for *S. fruticosa* polyphenol extraction by the CDs tested.

| Cyclodextrin | Maximum Predicted Response (YTP, 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 |

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_{a} 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 YTP, but it was not clear whether the presence of any CD used could affect YTP. 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 YTP, highlighting the importance of the CDs used as aqueous extraction boosters. The highest difference in YTP 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 | YTP (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 dH2O     | 40.59 ± 1.01        | -                | 93              | 3.75 |
| HP-β-CD           | 50.52 ± 1.19        | 1.40             | 100             | 3.90 |
| Buffered dH2O     | 40.76 ± 1.02        | -                | 100             | 3.90 |
| m-β-CD            | 54.25 ± 1.35        | 1.40             | 94              | 4.54 |
| Buffered dH2O     | 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]:

...
\[ Y_{TP(t)} = \frac{Y_{TP(s)}^2kt}{1 + Y_{TP(s)}kt} \]  

(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 \(Y_{TP(0)}/t\), is defined as follows:

\[ h = kY_{TP(s)}^2 \]  

(5)

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\(^{-1}\)) 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\(^{-1}\), 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 AAR (1112.51 μmol DPPH g\(^{-1}\) dm\(^{-1}\)), followed by HP-β-CD (836.17 μmol DPPH g\(^{-1}\) dm\(^{-1}\)) and β-CD (824.08 μmol DPPH g\(^{-1}\) dm\(^{-1}\)). The results for PR 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 $C_{CD}$, $R_{LS}$, and pH.

| $T$ (°C) | $k$ ($\times 10^{-3}$) (g mg$^{-1}$ min$^{-1}$) | $h$ (mg g$^{-1}$ min$^{-1}$) | $Y_{TP50}$ (mg GAE g$^{-1}$) |
|----------|---------------------------------|-----------------|-----------------|
| $\beta$-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-$\beta$-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-$\beta$-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 |

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

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]:

$$k = k_0 + ae^{-bT}$$  \hspace{1cm} (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-$\beta$-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]:

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

(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 6.** Diagram illustrating the AAR and PR 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 |
|-----------|---------------------|
|           | k₀ (×10⁻⁶) | a (×10⁻⁵) | b   | R²  | 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].

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

| No | Rt (min) | UV/Vis (λ<sub>max</sub>) | [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       |
| 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          |

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

| Extract                | Yield (mg g<sup>-1</sup> 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. fruticosa*, 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. 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, in order to study the effect of structural features on polyphenol extractability.

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**Nomenclature**

- **AAR** antiradical activity (µmol DPPH g⁻¹)
- **CCD** 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_{e1}** 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_{LS}** 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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