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

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Abstract

In a previous study, it was 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* Mill. (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. In this frame, this study was undertaken to generate extracts of *S. fruticosa* with LA-SCDB15 (a 77/23 w/w mixture of DES with water) and examine their stability in this solvent. *S. fruticosa* extracts exhibited remarkable stability under both accelerated and long-term conditions, and the antiradical activity and the ferric-reducing power of the extracts were shown to suffer virtually trivial modifications. 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.

Introduction

A high number of populations around the globe routinely utilize medicinal and aromatic plants (MAPs) as folk medical agents, but also food, for centuries. Currently, sound scientific data form a solid ground to support the pharmacological and nutritional attributes of MAPs and substantiate health claims and functionality (Anton *et al*. 2019). The ongoing interest for MAPs and MAP-based commodities derives from consumer demands for natural products with functional properties, which has stimulated a large development of neoteric botanical-based ingredients for foods, pharmaceutical and cosmetics (Campa and Baron 2018; Colombo *et al*. 2020). *S. fruticosa* Mill., otherwise known as *S. triloba* (Greek or Cretan sage) is a Lamiaceae species widely distributed in the East Mediterranean. Several bioactivities have been attributed to this medicinal plant, which

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are mainly due to its polyphenolic substances (Exarchou *et al*. 2015; Sarrou *et al*. 2016). Yet, to-date the implementation of green extraction methodologies to produce of polyphenol-enriched extracts from *S. fruticosa* is very limited.

Deep eutectic solvents (DES) are novel liquids composed of inexpensive, benign and reusable materials, embracing natural compounds, such as organic acids and their salts, polyols, amino acids etc. Usually, DES are composed of two constituents, one that functions as hydrogen bond acceptor (HBA) and another one as hydrogen bond donor (HBD), while DES synthesis is of low-cost, simple, and fast. Major intrinsic characteristics of DES are the tunability of composition to achieve water (im)miscibility, the low vapor pressure, and the absence of flammability, which make DES ideal solvents for a range of green processes (Espino *et al*. 2018). By virtue of their peculiar properties, DES have currently become the choice of preference for the effective extraction of numerous natural products, but important issues pertaining to extract stability in DES are presently poorly addressed.

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*. 2020b). 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.

Experimental

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 (≥ 98 %), gallic acid (97 %), sodium carbonate (≥ 99.5 %), ascorbic acid (≥ 99 %), sodium acetate trihydrate (≥ 99.5 %), luteolin 7-*O*-glucoside (≥ 98 %), 2,2-diphenylpicrylhydrazyl (DPPH) (95 %) and chlorogenic acid (≥ 95 %) were from Sigma-Aldrich (Darmstadt, Germany). Folin-Ciacalteu reagent was from Merck (Darmstadt, Germany). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) (98 %), acetic acid, methanol 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*. 2020b). The DES was used as aqueous mixture (77/23 w/w).

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*. 2020a) and stored under refrigeration (4 °C).

Preparation of polyphenol extracts

Extraction of *S. fruticosa* was performed using the optimized process, as reported elsewhere (Grigorakis *et al*. 2020b). 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 °C). 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 (Ss) of 900 rpm, at 80 °C, 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.

Determination of the antiradical activity (A_{AR})

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 polyphenols in the extract were determined using the following Eq. 1:

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

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 is the A_{515} at t = 0 and A_f is the A_{515} at t = 30 min. Results were expressed as µmol DPPH per mg total polyphenols. For all measurements, an HP 8452A diode-array spectrophotometer was used.

Determination of the ferric-reducing power (P_R)

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 extract and the mixture was heated up at 37 °C, 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).

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 extract, 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.

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 °C. 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 gradient program 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 (2 × 10^{-5} bar), collision pressure at 1.5 mTorr (2 × 10^{-6} bar) and capillary temperature 300 °C. Quantification was performed using a calibration curve of luteolin 7-*O*-glucoside (5 – 1,500 µg.L^{-1}, R^2 = 0.9982), chlorogenic acid (50 – 1,500 µg.L^{-1}, R^2 = 0.9986) and rosmarinic acid (50 – 3,000 µg.L^{-1}, R^2 = 0.9985). All standard solutions were prepared in HPLC grade methanol.

Accelerated stability test

A volume of extract (20 mL) was placed in a glass vial and heated up at 50, 60, 70, 80 and 90 °C 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.

Long-term stability test

Equal volumes of extract (20 mL) were transferred into glass vials and stored in the fridge (7 °C), on the bench (23 ± 2 °C) and in a water bath adjusted at 40 °C, 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.

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).

Results and Discussion

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 °C) to severe (90 °C) heating, for 240 min, and thus to draw conclusions regarding extract stability in LA-SCDB15. The results of the test are depicted in Fig. 1. Over the range 50 to 80 °C, 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 °C. 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 °C varied between0 and 3.1 %. This finding strongly suggested that LA-SCDB15 used to produce the extract, provided exceptional stability.

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 Fig. 2, the A_{AR} of the extract stored at 7 °C exhibited a significant decline by 39.9 %,

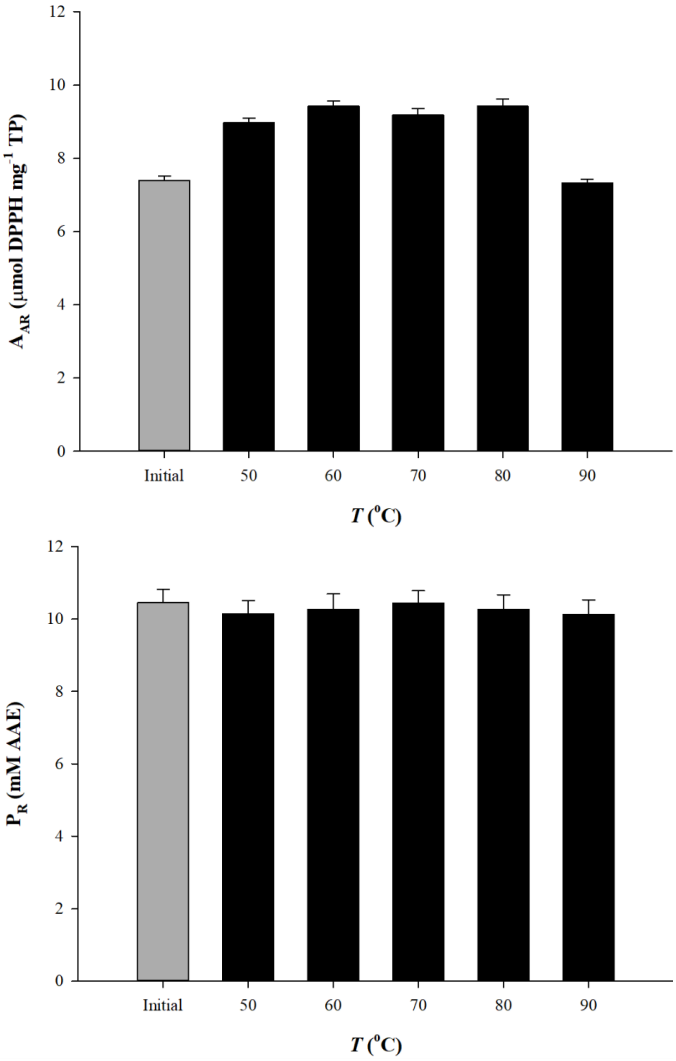

Fig. 1. Comparison of A_{AR} (upper plot) and P_R (lower plot) of *S. fruticosa* extracts, undergone no treatment (initial) and after treated at 50 – 90 °C, for 240 min.

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 °C) 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 °C 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. 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}.

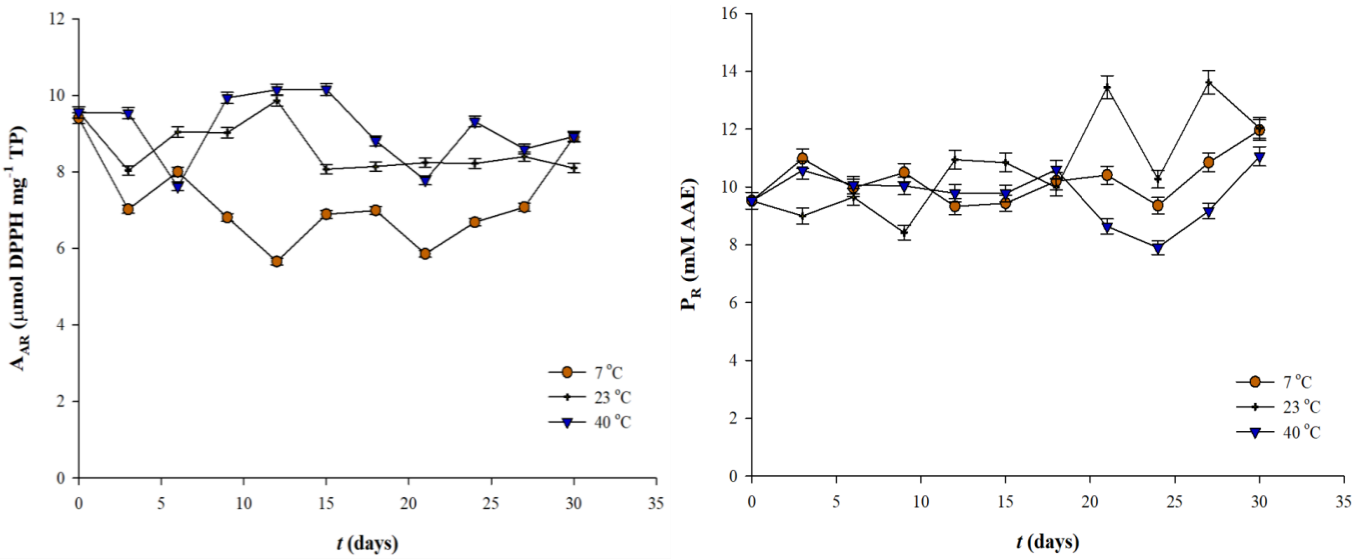

Fig. 2. Monitoring of A_{AR} (left plot) and P_R (right plot) of *S. fruticosa* extracts, stored at 7, 23 and 40 °C, for 30 days.

The pattern observed for the samples stored at 7 and 40 °C 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 °C showed rather large variations between day 18 and day 30, while its final level (day 30) was 12.04 mM AAE. The difference amongst P_R values at day 30 and the initial extract (9.53 mM AAE) was 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 °C), which obeyed pseudo-first order kinetics (Karageorgou *et al*. 2018). At 50 °C, 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 °C). The presence of methyl *β*-cyclodextrin delayed the progression of the phenomenon, yet at 50 °C 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 °C, 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 °C, although in this case too, P_R was stable and fluctuated within

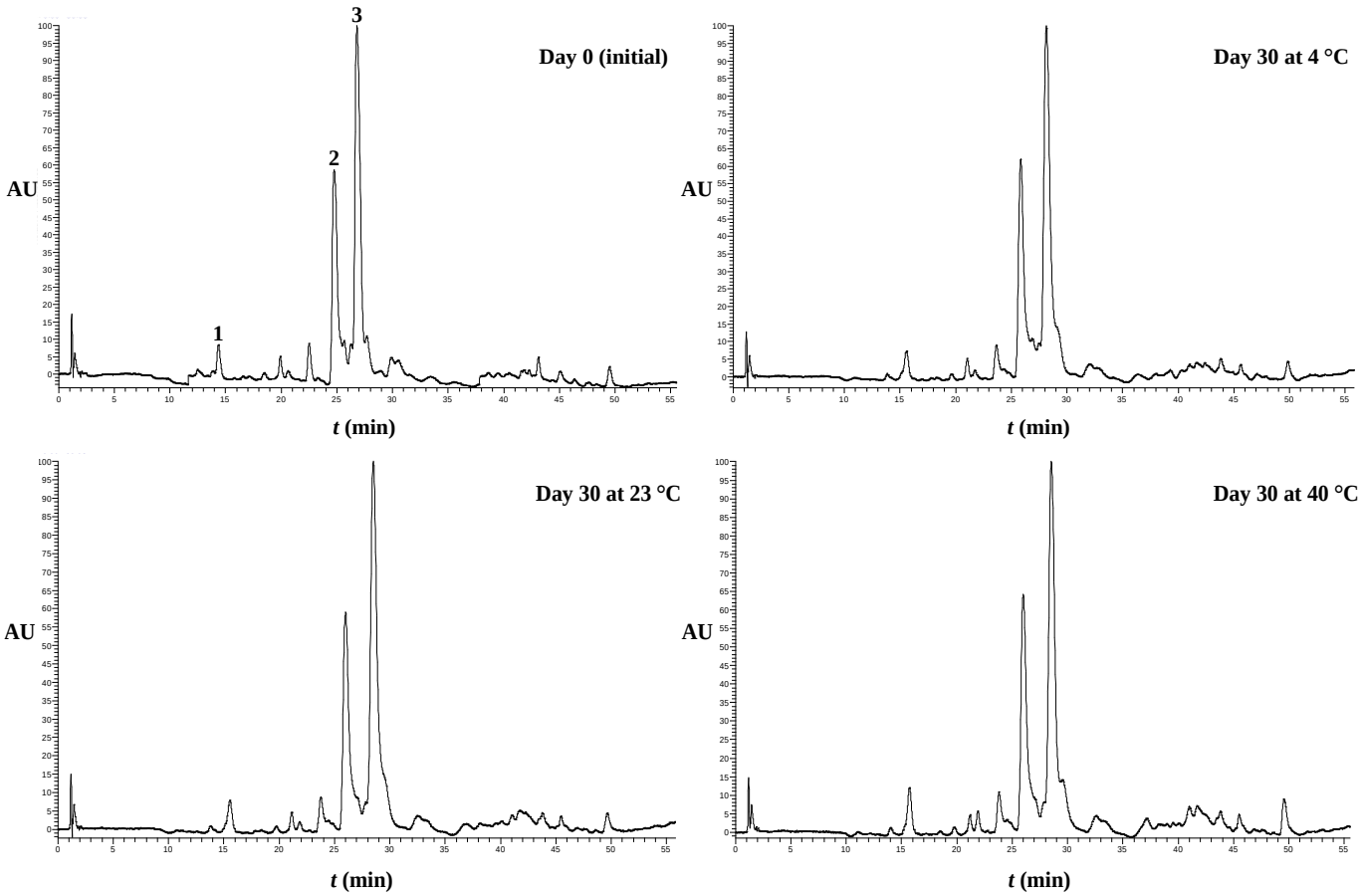

Fig. 3. Chromatograms of *S. fruticosa* extracts, obtained at 330 nm, illustrating the effect of extract storage at different temperatures after 30 days, on the major polyphenolic compounds. Peak assignment: 1– chlorogenic acid; 2 – luteolin 7-*O*-glucuronide; 3 – rosmarinic acid.

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 for other extracts.

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 °C), had identical polyphenolic profile and no major differences were seen (Fig. 3).

This outcome indicated neither 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 1). Storage at 7 °C resulted in a by 5.6 % decrease in the sum

Table 1. Quantitative data on changes occurred on major *S. fruticosa* polyphenols, after storage of extracts at different temperatures, after 30 days. Values given (mg.g^{-1} dry mass) represent means ± standard deviation.

Compound	Storage temperature			
	Initial	4 °C	23 °C	40 °C
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	19.6	18.59	19.48

of compounds, while at 23 and 40 °C 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.

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 °C, 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 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.

Conflict of Interest

The authors declare that they have no conflict of interest.

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