Single-step extraction of bioactive compounds from cruciferous vegetable (kale) waste using natural deep eutectic solvents

Sze Ying Lee [a] , Yen Nan Liang [a,b], David C Stuckey [c,d], and Xiao Hu [a,b*]

[a] Environmental Chemistry and Materials Centre, Nanyang Environment & Water Research Institute, Nanyang Technological University, 1 Cleantech Loop, Clean Tech One, Singapore 637141, Singapore

[b] School of Materials Science and Engineering, Nanyang Technological University, 639798, Singapore

[c] Advanced Environmental Biotechnology Centre, Nanyang Environment & Water Research Institute, Nanyang Technological University, Singapore 637141, Singapore

[d] Department of Chemical Engineering, Imperial College London, London SW7 2AZ, UK

E-mail addresses:

Sze Ying Lee (szeying.lee@ntu.edu.sg)

Yen Nan Liang (ynliang@ntu.edu.sg)

David C Stuckey (d.stuckey@imperial.ac.uk)

Xiao Hu (asxhu@ntu.edu.sg)

*Corresponding author

Tel: (65) 6790-4610

ABSTRACT

Cruciferous vegetables such as kale contain various health-promoting phytochemicals, and leaves rejected from harvesting could be a valuable source of phytochemicals. Conventional organic solvents used for the recovery of these bioactive metabolites are hazardous, and therefore more benign equivalents are sought. This work explores the use of natural deep eutectic solvents (NADESs) with different hydrophilicity/hydrophobicity to recover polyphenols, carotenoids and chlorophylls from kale waste. Enhanced extraction of polyphenols was achieved by the aqueous solutions of hydrophilic glycerol-based NADESs, with yields of up to 2.2-fold higher than just using methanol. The antioxidant capacity of the aqueous extracts showed a strong correlation with their total phenolic contents. The best solvent in extracting polyphenols (glycerol:betaine at a molar ratio of 3:1) was further investigated to optimise its process conditions. The optimised extract provided the greatest stability of the bioactive polyphenols by retaining 91.7 and 88.6% of the original contents after 30 days of storage at 4 and 25 °C, respectively. Furthermore, this extraction approach was integrated with ethyl acetate, a bio-based solvent, to promote the simultaneous recovery of polyphenols (16.83 mg GAE (gallic acid equivalents) g^{-1} DW (dry weight)), carotenoids (0.91 mg g^{-1} DW) and chlorophylls (7.86 mg g^{-1} DW) from kale waste. After extraction, the immiscible aqueous NADES- and ethyl acetate-phases rich in polyphenols and carotenoids/chlorophylls, respectively, can be easily separated for different applications.

KEYWORDS: Extraction; natural deep eutectic solvents; ethyl acetate; kale waste; phenolic compounds; carotenoids.

1. Introduction

Food loss and waste have been serious global problems for many years, and are on an upward trend in many countries [1]. For instance, around 817,000 tonnes of food were wasted in Singapore in 2021, which was 23% higher than in 2020; almost half this food waste was fruit and vegetables [2]. Perishable produce usually suffers from massive losses during the post-harvest stage before reaching the supermarket shelves. Aside from post-harvest spoilage, large quantities are rejected due to imperfections in size, shape or colour when undergoing aesthetic screening for sale in supermarkets [3]. This loss results in adverse impacts on both the economy and the environment. Most rejected produce discarded due to their poor aesthetic appeal usually possess identical nutritional compositions and food safety as the saleable products. All this waste should be upcycled into higher-value products that can be fed back into the supply chain to achieve circularity and sustainability in agricultural production.

In particular, cruciferous vegetables such as kale (*Brassica oleracea* var. *acephala*), which is gaining popularity as a nutrient-dense "superfood", contain exceptionally high levels of phytochemicals that bring immense health benefits beyond basic nutrients [4]. Kale leaves rejected by cosmetic screening represent an attractive source of phytochemicals that could potentially serve as natural antioxidants, pigments and functional ingredients in cosmetic and food applications [5]. Indeed, kale is rich in flavonoids (e.g., quercetin and kaempferol) and contain a great number of antioxidants [6-8]. The total phenolics in freeze-dried kale were 14.03 mg g^{-1}, higher than either spinach (11.47 mg g^{-1}) or broccoli (8.33 mg g^{-1}) [9]. Polyphenols are powerful antioxidants that can relieve oxidative stress by radical scavenging activity (RSA), and thus reduce the risk of chronic degenerative illnesses [10]. In addition, lipophilic pigments such as carotenoids and chlorophylls, which can be found in kale, are proven to exert positive effects on inflammatory behaviour, neuroprotection and the reduction of coronary and eye disorders [11, 12]. Based on its health-promoting effects, kale extract has

been used in the formulation of cosmetics, nutraceuticals [13, 14] and pharmaceutical products [15, 16]. Clinical studies showed that the intake of an oral supplement of carotenoid-rich curly kale extract, with a daily dose of 4.45 mg total carotenoids, increased the skin's RSA and lipids [14]. Furthermore, a daily intake of carotenoids of 1.65 mg was also proven to avoid ageing-related collagen I degradation in the dermis [17]. Apart from work on the assessment of its efficacy, recovering kale extract is another critical aspect, which directly influences the level of acceptance by regulatory authorities and consumers, and its economic viability.

The isolation of bioactive compounds from vegetable waste has been addressed in the past through technologies that involve petroleum-derived volatile organic compounds (VOCs). Methanol, acetone, and their mixtures are often used to separate polyphenols from food waste [18, 19], whereas carotenoids are mostly extracted by hexane for food and pharmaceutical purposes. VOCs above certain concentration levels are detrimental to the environment and human health. Besides traditional extraction, advanced techniques like supercritical fluid [20] and microwave-assisted extraction [21] have been studied; however, they may not be economically viable on an industrial scale due to the use of high-cost specialised equipment. It would therefore seem important to develop scalable, sustainable, and safe extraction processes using less harmful solvents.

Natural deep eutectic solvents (NADESs) have been proposed as environmentally benign substitutes for hazardous VOCs [22]. They are liquids composed entirely of plant-based primary metabolites such as amino acids, sugars, sugar alcohols and organic acids [23]. In addition to their non-volatility and biodegradability, NADESs have a high-solubilising capacity for many natural products, making them suitable extraction media. Moreover, the natural constituents of NADESs make it possible for their direct use in extracts for food, pharmaceutical and cosmetic applications, hence simplifying the product polishing step [24]. Furthermore, the predominantly polar NADESs are superior in extracting polar metabolites,

while hydrophobic NADESs can be used in isolating ergosterol [25] and carotenoids such as lutein [26], astaxanthin [27] and β-carotene [28] from various matrices. Most of these studies often focus on the recovery of a specific group of compounds (either hydrophilic or lipophilic); there is still limited information about the capacity of hydrophilic/hydrophobic NADESs to extract different classes of bioactive metabolites from kale waste. The development of sustainable recovery processes is needed to efficiently valorise kale waste for the production of natural antioxidants and pigments. Hence, this work evaluated the capacity of different NADESs to extract polyphenols, carotenoids like β-carotene and lutein, and chlorophylls from kale waste. All NADES precursors selected were renewable and low-cost ingredients that are allowed for food and cosmetic use. In particular, eight hydrophilic NADESs were prepared by pairing glycerol with betaine, sorbitol, xylose, glucose, fructose and urea at appropriate molar ratios [29, 30]. In contrast, different combinations of terpenes including DL-menthol, thymol and fenchyl alcohol at appropriate molar ratios formed the four hydrophobic NADESs studied in this work [26]. It is worth mentioning that despite the ability of organic acids to form NADESs, they were not selected because their acidic nature might induce instability in food and cosmetic formulations [31]. Furthermore, another extensively studied NADES constituent, i.e., choline chloride, was also not used in this work due to its prohibition in cosmetic products under European law (EC No 1223/2009). After initial screening of solvents, the antioxidant activity of the extracts was analysed via the DPPH RSA assay. Next, the extraction conditions of the most promising solvent for polyphenols were evaluated, and the stability of the polyphenol-rich extract was assessed during storage. Finally, to maximise the valorisation potential of kale waste, a two-step sequential process, as well as a single-step integrated approach, were evaluated to recover multiple molecules of interest from the kale waste. Figure 1 illustrates schematically the main steps applied in this work.

2. Material and methods

2.1. Chemicals

Gallic acid (certified reference material), β-carotene (pharmaceutical secondary standard), lutein (pharmaceutical secondary standard), chlorophyll *a* from spinach (≥ 85%), chlorophyll *b* from spinach (≥ 90%), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (≥ 97%), glycerol (≥ 99%), betaine (≥ 99%), D-sorbitol (≥ 98%), D-(+)-xylose (≥ 99%), D-(+)-glucose (≥ 99.5%), D-(−)-fructose (≥ 99%), urea (≥ 99%), DL-menthol (≥ 95%), thymol (≥ 98.5%), fenchyl alcohol (≥ 97%), triethylamine (≥ 99%), tetrahydrofuran (THF) (⩾ 99%) and Folin & Ciocalteu′s phenol reagent were purchased from Sigma-Aldrich. Methanol (LCMS grade, ≥ 99.9%), acetonitrile (LCMS grade, ≥ 99.9%) and ethanol (HPLC grade, ≥99.8%) were supplied by Fisher Scientific. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (≥ 95%) was acquired from Alfa Aesar. All the chemicals were used directly without further purification. Ultra-pure water was prepared by the Millipore Milli-Q water purification system.

2.2. Kale waste

Kale waste was provided by Sustenir Agriculture (Singapore). Curly kale leaves used in this work did not meet commercial quality standards and were intended to be discarded as waste by the urban farming company. The kale waste was cleaned, freeze-dried, ground in a laboratory ball mill, sieved to obtain a powder size of < 0.2 mm and stored in a sealed container at 4 °C until further use.

2.3. Preparation of natural deep eutectic solvents (NADESs)

NADESs were prepared by mixing the respective precursors at certain molar ratios in glass vials with constant heating (maximum at 80 °C) and stirring until a clear homogeneous liquid was formed. They were kept at room temperature and observed to have no precipitate formed in the liquid. All abbreviations of NADESs and their compositions used in this work are detailed in Table 1.

2.4. Viscosity and pH measurement

Viscosities of all hydrophilic NADESs containing 30% (w/w) water and neat hydrophobic NADESs, respectively, were determined using a modular compact rheometer (Anton Paar MCR 102, Germany) fitted with a cone and plate measuring geometry with 50 mm of diameter (CP50-1). The gap between the cone and plate was set as 0.095 mm and the temperature of the system was controlled by a Peltier temperature device (P-PTD200). All measurements were performed at 25 °C and a constant shear rate of 10 s^{-1} for 10 s. Final viscosity was obtained as the average of the results. All aqueous solutions of hydrophilic NADESs containing 30% (w/w) water were measured for their pH using a pH meter (Mettler Toledo, Singapore) at 25 °C.

2.5. Solid-liquid extraction

The lyophilised kale waste powder was subjected to solid-liquid extraction (SLE) using a series of solvents at a solid-liquid ratio (SLR) of 1:40 (g mL^{-1}) at 150 rpm and 25 °C for 30 min. All hydrophilic NADESs tested were in hydrated form with 30 wt% water added, while neat hydrophobic NADESs were used. The mixture was vortexed at 3000 rpm for 30 s. All studies were performed under reduced lighting to prevent the degradation of light-sensitive compounds. The mixture was then centrifuged at 12000 g for 10 min. The supernatants were collected and filtered with PTFE syringe filters (0.45 µm, 25 mm) before quantitative analysis.

2.6. Process optimisation for the extraction of polyphenols

After preliminary screening, the best solvent to extract polyphenols was identified and the process conditions were further investigated by one-factor designs. The effects of process parameters including SLR (1:10, 1:15, 1:20, 1:30 and 1:40), temperature (25, 45 and 65 °C), solvent concentration (50, 60, 70 and 80%) and time (30 and 60 min) were assessed. For comparison purposes, ultrasound-assisted extraction was also conducted using an ultrasonic bath (Elma Elmasonic P, Germany) operating at a constant frequency of 37 kHz and 100% output power. The ultrasonic bath was at 25 °C at the beginning of the process and increased

to 30 and 38 °C after 30- and 60-min operation, respectively. The supernatants were collected by centrifugation at 12000 g for 10 min and filtered before assaying for total phenolics.

2.7. Stability of the extract over time

The polyphenol-rich extracts obtained with the best solvent, and several reference solvents at their optimal conditions, were analysed for stability for 30 days at 25 and 4 °C excluding light. The residual total phenolics in the extracts were determined at least once a week. The results are presented as the residual concentration of total phenolics compared to the total phenolics in the original extract obtained with water (before storage), as described in Eq. 1.

$$\text{Relative residual concentration} = \frac{\text{Total phenolics in the extract at the time of storage}}{\text{Total phenolics in the original extract obtained with water}} \times 100\% \quad (1)$$

2.8. Sequential and integrated extraction approaches

Sequential processes were designed to recover polyphenols, carotenoids and chlorophylls from kale waste. In Route 1, the lyophilised kale powder was treated with Gly^3:Bet, at the optimal conditions (25 °C, 30 min, SLR of 1:20 and concentration of 70%) to recover polyphenols. The polyphenol-rich supernatant was collected by centrifugation at 12000 g for 10 min and measured for total phenolics. Next, the residual pellet was subjected to the second SLE using ethyl acetate; and the supernatant obtained after centrifugation was quantified for carotenoid and chlorophyll contents. Inversely, for Route 2, kale waste was first treated with ethyl acetate followed by subsequent extraction using Gly^3:Bet. To simplify the processes, an integrated strategy (Route 3) was attempted by employing ternary mixtures of 1:1 (v/v) of Gly^3:Bet-water mixture (30:70 w/w) and ethyl acetate under the same process conditions. After centrifugation, the top (ethyl acetate-) and bottom (aqueous Gly^3:Bet-) layers were collected to quantify the target compounds. The constituents in the top and bottom phases of the system were assessed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy (JEOL ECA400).

2.9. Modified integrated extraction approach with wet kale waste paste

The applicability of the integrated extraction approach for wet kale waste was investigated. The kale waste was clean and cryogenically ground (SPEX 6875 Freezer/Mill, U.K.), forming a wet paste containing 89.6% water. The wet paste was further concentrated to around 75% water content in order to maintain the SLR of the extraction process. Considering the water in the kale waste paste as the substitute for water in the solvent system, the integrated extraction approach was modified with the use of only Gly^3:Bet and ethyl acetate. The extraction was carried under the same process conditions described in Section 2.8.

2.10. Total phenolic content assay

Total phenolics were estimated as gallic acid equivalents (GAE) using the Folin-Ciocalteu protocol described by Singleton et al. [32], with slight modifications. 25 µL of diluted sample was mixed with 200 µL of water followed by the addition of 25 µL of 25% (v/v) Folin-Ciocalteu reagent. After 5 min, an aliquot of 25 µL of 10% (w/v) sodium carbonate was added, and the reaction mixture was shaken gently and incubated in the dark at 25 °C for 60 min. The total phenolics in the extract were determined calorimetrically by measuring the absorbance at 765 nm with a microplate reader (Tecan Infinite M200 Pro, Singapore) based on the calibration curve of gallic acid. To eliminate the effect of the solvents, a blank control system for each sample was prepared under the same conditions. Results were documented as mg gallic acid equivalent per g dry weight of kale waste (mg GAE g^{-1} DW). For the hydrophobic solvents studied, the extract collected was subjected to liquid-liquid extraction (LLE) by adding an equivalent volume of water. The mixture was shaken vigorously, and the aqueous layer collected after phase separation by centrifugation was assayed for total phenolics. The total phenolics in the extracts of Men:Thy, Men:Thy^2 and Thy:Fen were not determined due to the interference with the assay's reagents.

2.11. Carotenoid (β-carotene and lutein) and chlorophyll (*a* and *b*) quantification

A reversed-phase high-performance liquid chromatographic (RP-HPLC) protocol was used to quantify β-carotene, lutein, and chlorophyll *a* and *b* in the extract. Separation and detection of these compounds was accomplished with an Agilent 1260 Infinity II LC system (Agilent Technologies, Singapore) equipped with a diode-array UV-Vis detector, set at 450 nm, on an Agilent ZORBAX Eclipse Plus C18 (3.0 mm ×150 mm, 3.5 μm) column with a 15 min isocratic elution method at 1.0 mL min^{-1}. The mobile phase was methanol and acetonitrile (9:1 v/v), with an aliquot of 0.1% (v/v) of triethylamine added to prevent both nonspecific adsorption and oxidation. The column was maintained at 25 °C, while the sample injection volume was 5 μL.

Each compound was identified based on the retention time of commercial standards. The stock solution of β-carotene was prepared by dissolving it in a small amount of THF stabilized with butylated hydroxytoluene (BHT) (1% (v/v) of total solvent volume) before diluting it with ethanol. Lutein and chlorophyll standard stock solutions were prepared using ethanol and methanol, respectively. The exact concentration of standard stock solutions was assessed spectrophotometrically (Shimadzu UV-1800, Japan) and corrected by applying the reported extinction coefficient, $\varepsilon_{1cm}^{1\%}$, of each compound at their respective wavelength: β-carotene ($\varepsilon_{1cm}^{1\%}$ in ethanol = 2529 cm^{-1} (g/100 mL)$^{-1}$ at 450 nm); lutein ($\varepsilon_{1cm}^{1\%}$ in ethanol = 2550 cm^{-1} (g/100 mL)$^{-1}$ at 445 nm); chlorophyll *a* ($\varepsilon_{1cm}^{1\%}$ in methanol = 799.5 cm^{-1} (g/100 mL)$^{-1}$ at 665 nm) and chlorophyll *b* ($\varepsilon_{1cm}^{1\%}$ in methanol = 424.8 cm^{-1} (g/100 mL)$^{-1}$ at 652 nm) [33, 34]. Results were expressed as mg per g dry weight of kale waste (mg g^{-1} DW).

2.12. DPPH radical-scavenging activity (RSA) assay

The antioxidant capacity of the extract was assayed using the DPPH method. 25 μL of diluted sample was mixed with 800 μL of 0.0072 mM methanolic DPPH solution. The mixture was shaken vigorously and left to stand in the dark at 25 °C for 30 min. RSA was estimated by

measuring the reduction of DPPH radicals, which was expressed as a percentage of DPPH discolouration using Eq. 2.

$$\text{RSA} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100\% \quad (2)$$

Where $\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{sample}}$ were the absorbance values of the control and sample, respectively, at 517 nm. The influence of the solvents was eliminated by preparing a blank control system under the same conditions. Results were converted and recorded in mg of Trolox equivalent per g dry weight of kale waste (mg TE g^{-1} DW).

2.13. Data analysis

Triplicate independent runs were performed for each condition, and the results were expressed as average ± standard deviation. A statistical significance analysis was conducted using one-way ANOVA analysis and accompanied by a post-hoc Tukey method for all pair-wise multiple comparisons. The level of statistically significance was set at $P < 0.05$. Before applying ANOVA, the Shapiro–Wilk test was used to confirm no violation of the assumption of normality in the dataset.

3. Results and discussion

3.1. Screening of solvents

The performance of hydrophilic and hydrophobic NADESs for the recovery of bioactive metabolites, particularly polyphenols, carotenoids (β-carotene and lutein) and chlorophylls (*a* and *b*), from kale waste after 30 min of stirring at 150 rpm and 25 °C was first screened. Because of the high viscosity of hydrophilic NADESs that might hinder their extraction ability [35], they were used in hydrated forms, i.e., with 30% water added. To facilitate the comparison, several reference solvents, including water (H_2O), methanol (MeOH), 70% aqueous methanol (70% MeOH), ethanol (EtOH), 70% aqueous ethanol (70% EtOH), 70% aqueous glycerol (70% Gly), *n*-hexane (Hex) and ethyl acetate (EtOAc), were tested under the same conditions. Figure 2A presents the results of yields of target compounds using different solvents. All hydrophilic

glycerol-based NADESs displayed selective extraction towards polyphenols, whereas lipophilic compounds were favourably extracted by hydrophobic terpene-based NADESs.

All aqueous solutions of glycerol-based NADESs, except for Gly:U, achieved higher yields of polyphenols compared to the reference solvents. This could be attributed to their strong interactions with NADES constituents which are good hydrogen bond donors and acceptors, as listed in Table S1 in the Supporting Information. Similarly, Cao and co-workers [36] correlated the solubility of several phytochemicals to their interactions with NADES through dipole-dipole and hydrogen bond formation. Hydroxycinnamic acid derivatives like ferulic, caffeic and sinapinic acid and flavonoids such as quercetin and kaempferol were the most common phenolic groups identified in kale leaves [37]. In this work, Gly^3:Bet gave the highest yield of polyphenols, which was around 2.2- and 1.3-fold higher than that of using MeOH and H_2O, respectively. Furthermore, glycerol-based NADESs coupled with sugar alcohols, namely sorbitol (Gly^3:Sor) and xylose (Gly^3:Xyl), demonstrated relatively good dissolution capacity towards polyphenols compared to their sugar counterparts, which were glucose (Gly^3:Glu) and fructose (Gly^3:Fru). Since the NADESs tested possess a common glycerol constituent, the variation of the yields among the solvents could be attributed to the different hydrogen bonding capacities of the second component. The extraction aptitude of glycerol-based NADESs for polyphenols from kale waste is in close agreement with the polarity of their constituent, as confirmed by the predicted logarithmic function of the octanol-water partition coefficient (log K_{OW}) obtained from ChemAxon: urea (log K_{OW} = −1.364) < fructose (log K_{OW} = −2.758) < glucose (log K_{OW} = −2.933) < xylose (log K_{OW} = −2.938) < sorbitol (log K_{OW} = −3.730) < betaine (log K_{OW} = −4.494). In contrast, no linear relationship was observed between pH/viscosity and yield based on the data shown in Table 1. Nevertheless, it was noted that the best extractant, 70% Gly^3:Bet, possesses relatively low viscosity (19.33 mPa·s) and almost neutral pH. In contrast, 70% Gly:U, which also has low viscosity, suffered

from poor yield, probably due to its alkalinity. Chagnoleau and co-workers [38] suggested that the yield of phenolic compounds from kiwifruit seems to be controlled by the solvent pH when the solvent viscosity was below 20 mPa·s, and the dissolution of phenolics was more favourable under acidic conditions.

As opposed to the NADESs studied, polar solvents such as MeOH, EtOH, 70% MeOH and 70% EtOH resulted in much more complex extract contents encompassing polyphenols, carotenoids and chlorophylls. Alcoholic solvents showed good extraction capacity for carotenoids and chlorophylls, however, relatively lower yields of polyphenols were obtained. Specifically, more polar MeOH (log K_{OW} = –0.77) extracted more polar lutein and chlorophylls but less non-polar β-carotene compared to EtOH (log K_{OW} = –0.31). The addition of water to polar solvent systems improved the ability of alcohols to dissolve polyphenols, but diminished the solubilisation of lipophilic compounds, especially β-carotene which is non-polar, as exhibited by 70% MeOH and 70% EtOH. On the other hand, moderately polar EtOAc (log K_{OW} = –0.31) presented a balanced ability to extract a wide range of lipophilic compounds from kale waste, from the most polar lutein to chlorophylls and non-polar β-carotene (their chemical structures are provided in Figure S1 in the Supporting Information). Lutein is a xanthophyll having a pair of hydroxylations in the terminal β rings, making it more polar than hydrocarbon β-carotene, and thus it was more extractable in polar solvents like EtOH and MeOH, but least soluble in non-polar hexane. On the other hand, a chlorophyll molecule consists of a hydrophilic porphyrin head and a long lipophilic hydrocarbon tail; it has an intermediate polarity between lutein and β-carotene. Chlorophyll *b*, which has an aldehyde group at the position 7-carbon, is slightly more polar than chlorophyll *a* that has a methyl group at the same position. It was noted that the pigment elution sequence in RP-HPLC followed the order of decreasing polarity of each pigment, as presented in the chromatograms of several representative sets of extracts depicted in Figure S1 in the Supporting Information.

All hydrophobic terpene-based NADESs studied in this work (i.e., Men:Thy, Men:Thy^2, Men:Fen and Thy:Fen) exhibited comparable extractability for β-carotene and lutein than hexane; but lower yields of lutein compared to that of EtOAc. It was observed that lutein and chlorophylls demonstrated similar extraction characteristics, probably because of their similar polarity. Similar findings were also reported by Derrien et al. [39] that lutein and chlorophylls can be extracted with the same level of solvent concentration and temperature. Although the yields of lutein and chlorophylls obtained using hydrophobic NADESs in this screening test were lower compared to reference solvents, this could probably be improved by optimising process conditions. Fan et al. [26] demonstrated that Thy:Fen achieved a higher yield of lutein from microalgae than EtOAc after stirring at 1200 rpm and 30 °C for 70 min, and the yield can be increased by elevating the process temperature to 60 °C.

3.2. Antioxidant potential of the aqueous extracts

Antioxidant capacity is one of the most important biological characteristics of an extract with respect to its applications. The antioxidant capacity of all aqueous extracts was characterised based on the measurement of its ability to scavenge the stable radical DPPH$^{\cdot}$, and these results are presented in Figure 2B. Interestingly, the results of antioxidant capacity followed the identical trend of total phenolics in the extracts, as demonstrated by the good correlation between them (R^2 = 0.9013, Figure 2C), revealing that the antioxidant activity determined by the DPPH method was governed primarily by polyphenols in the extracts. On the other hand, carotenoids and chlorophylls in methanolic and ethanolic extracts did not seem to exert a synergistic effect to significantly increase the antioxidant capacity of the respective extracts, although the antioxidant behaviours of carotenoids and chlorophylls are well documented [40, 41]. Similar findings were reported in several works involving extracts derived from fruit [42] and vegetables [43, 44], and even microalgae [45]. Thaipong and co-workers [46] observed that the antioxidant capacity of methanolic guava extract was strongly correlated with total

phenolics, but had a negative relationship with total carotenoids. Likewise, Cervantes-Paz et al. [47] concluded that the antioxidant activity of a pepper extract was not governed by its carotenoid and chlorophyll contents in view of the poor relationship between antioxidant activity and the pigment concentration (R^2 = 0.0024 – 0.1332).

Polyphenols have been recognised as the dominant antioxidants in Brassica crops [48]; they displayed higher antioxidant activity than vitamins and carotenoids, as proven by in vitro studies [49, 50]. Wu et al. [51] reported that water-soluble antioxidants in Brassica vegetables contributed to > 89% of total antioxidant capacity, based on the data obtained using the oxygen radical absorbance capacity (ORAC) assay. Additionally, the antioxidant capacity of individual flavonoids isolated from kale has been validated in the work of Fiol et al. [52], which showed that the quercetin derivatives with a catechol structure contributed to high antioxidant activity. Moreover, other work conducted by Lanfer-Marquez et al. [53] stated that the chlorophylls extracted from fresh spinach leaves displayed extremely low RSA (<12%) when compared to Trolox which scavenged around 80%, based on the DPPH method. In this work, Gly^3:Bet appeared to be an excellent candidate to produce a polyphenol-rich extract with remarkable antioxidant properties for applications in the cosmetic, food and pharmaceutical industries. In addition, glycerol is frequently used as a humectant or moistener in cosmetics [54], besides being widely used as an excipient in pharmaceutical formulations, such as providing lubrication and smoothness in many cough syrups and other drugs [55]. Furthermore, betaine is commonly consumed as a dietary supplement and is often used in personal care products [56]. Therefore, Gly^3:Bet was selected for further process optimisation work.

3.3. Extraction of polyphenols using Glycerol: Betaine

Based on the NADES selected, single-factor experiments were conducted to evaluate the effect of process parameters (i.e., SLR, solvent concentration, temperature, and time) on maximising the yield of total phenolics. The influence of the SLR was examined first, and the results are

presented in Figure 3A. There was no significant difference in the yields when SLRs between 1:40 to 1:20 were used. However, the yield of polyphenols decreased significantly with a further increase in SLR; to use less solvent, a SLR of 1:20 was chosen as optimal and used for subsequent studies.

One of the main drawbacks of NADESs is their high viscosity compared to traditional organic solvents. This reduces the diffusion of the solute of interest, and hence reduces the mass transfer rate, which in turn leads to slower recovery performance [35]. Given this constraint, the application of NADESs as an extraction media often involves the addition of water. An appropriate dilution is important to reduce the solvent's viscosity and at the same time retaining their eutectic properties. Hence, the effect of the solvent concentration was studied in addition to the extraction temperature and time. The results depicted in Figure 3B clearly show that an increase in the temperature has a deleterious effect on the yields of total phenolics, and the impact was more pronounced at low concentrations of Gly^3:Bet, i.e., 50%. Additionally, when heat was supplied during extraction, a prolonged processing time of 60 min led to markedly reduced yields. The results implied that the polyphenols in kale are very vulnerable to higher temperatures, which is in good agreement with the findings reported by Lafarga et al. [57] that thermal processing such as steaming (100 °C, 15 min) and *sous-vide* (80 °C, 15 min) dramatically reduced the total phenolics in the kale leaves. It is worth noting that the detrimental impact of high temperature, i.e., at 45 and 60 °C, was less at high solvent concentrations, revealing that the Gly^3:Bet exerted a protective effect on polyphenols from degradation. However, the protective effect of Gly^3:Bet vanished at 50%, indicating that this is the limit where its intermolecular interactions are minimised. A similar phenomenon was also observed by Dai et al. [58] where the addition of 50% (v/v) water to lactic acid-glucose provoked a dramatic change in structure and polarity, and this was probably due to the rupture of hydrogen bonding between lactic acid and glucose.

From Figure 3B the optimal conditions for separating polyphenols from kale waste were at 25 °C for 30 min using 70% Gly^3:Bet, with yields comparable to that obtained with ultrasound-assisted extraction. Hence, with the chosen conditions and NADES as an extractant, process intensification using sonication is not necessary. Sonication may also be undesirable due to the generation of localised heating that could destroy thermo-sensitive products, as observed when the process duration was extended to 60 min. Dai et al. [22] also observed that simple mechanical stirring was much more efficient than sonication when extracting anthocyanins from *Catharanthus roseus* using a NADES based on lactic acid and glucose.

3.4. Stability of polyphenols in the extract over time

The preservation ability of the solvent system is important for product stability. The stability of polyphenol-rich extracts obtained with 70% Gly^3:Bet and several reference solvents after extraction was investigated by periodically measuring their residual phenolic contents during storage in the dark at 25 and 4 °C for 30 days. These results are expressed in terms of the relative residual concentration of total phenolics in the extracts compared to the total phenolics in the original extract obtained with water (Figures 4A and B). It can be clearly seen that polyphenols degraded faster at 25 °C than at 4 °C in all solvents tested. Rapid deterioration of polyphenols was found in water, in which > 50% and close to 30% of the original contents were lost after being stored at 25 and 4 °C for 30 days, respectively. In contrast, 70% Gly^3:Bet provided the greatest stability of the bioactive polyphenols by retaining 91.7 and 88.6% of the original contents after 30 days of storage at 4 and 25 °C, respectively (see Figure S2 in the Supporting Information). Likewise, 70% EtOH resulted in comparable performance maintaining the stability of polyphenols over time, however, the polyphenols extracted with 70% EtOH were the lowest amongst the solvents studied. The stabilisation of NADESs on bioactive compounds has also been noted for cyanidin [22] and catechin [59]. Dai et al. [22] correlated this with the extensive interactions between the biomolecules and NADES

constituents that consequently reduces oxidative degradation. In addition, a transmission electron microscopy (TEM) study by Ling et al. [60] found that the antioxidants from fruit waste of *Mangifera pajang* agglomerated in the centre of NADES molecules composed of choline chloride:ascorbic acid, forming a nano-scale cluster that protects the biomolecules.

All extracts, except for 70% EtOH, appeared orange due to the presence of polyphenol like flavonoids, while an abundance of chlorophylls led to a bright green colour in the 70% EtOH extract (Figure 4C). Consistent with the stability test, the fastest destabilisation was observed in the aqueous extract. It started to become turbid accompanied by visible colour fading after storing at 25 °C for 2 days, and precipitates developed over time during storage, although the degradation slowed down when the extract was kept at 4 °C. Similarly, the 70% Gly extract rapidly turned cloudy too, with a gradual colour change from orange to pale yellow over time (Figure S3 in Supporting Information). It is frequently reported that a higher degradation of the polyphenolic structure in the product was accompanied by an alteration in the product's colour [61]. In addition, a colour change from bright green to brownish olive green was seen in the 70% EtOH extract, with the concomitant formation and sedimentation of green particles, probably due to the conversion of chlorophylls to pheophytins and pyropheophytins [61]. Chlorophyll pigments are vulnerable to degradation during storage, as shown by Ahmadi and co-workers [62] where chlorophylls *a* and *b* extracted from alfalfa by enzymatic and ultrasound methods gradually deteriorated during storage at -18, 4 and 25 °C for 45 days. In contrast, a 70% Gly^3:Bet extract that was stored at 4 °C remained clear and had the least colour change throughout the storage period, confirming the good preservation capacity of the solvent system. The stable polyphenol-rich extract could be readily used in cosmetic or pharmaceutical formulations since the solvent constituents are active and compatible ingredients.

3.5. Sequential and integrated recovery of bioactive compounds

To promote the sustainable and efficient valorisation of kale waste, recovery of multiple value-added products is desirable. From Figure 2A considerable amounts of carotenoids like β-carotene (up to 0.45 mg g^{-1}) and lutein (up to 0.59 mg g^{-1}) are found in kale waste, compared to other wastes such as paprika leaves with 0.23 mg g^{-1} lutein [63]. Furthermore, chlorophyll is a well-recognised natural and permitted food colourant (European standard no. E140) [64]. Therefore, to further valorise the waste, a second separation unit was proposed to recover carotenoids and chlorophylls after the treatment with 70% Gly^3:Bet (designated as Route 1). Hence, EtOAc, a bio-based solvent, was selected to extract lipophilic pigments owing to its high extraction potential, while another approach with an inverse sequence (Route 2) was also investigated. Finally, an integrated method using pseudo-ternary mixtures of aqueous Gly^3:Bet and EtOAc (Route 3) was explored, forming a liquid-liquid extraction (LLE) biphasic regime composed of the top (EtOAc) and bottom (aqueous Gly^3:Bet) liquid phases. In this work, the Gly^3:Bet can be treated as pseudo-pure species since its constituent ratio was maintained after LLE formation, as validated by the ^{1}H NMR results (Figure S4 in the Supporting Information) demonstrating that none of the individual constituents partitioned into the EtOAc-phase.

From the results of these three approaches (Figure 5A), the yield of polyphenols using 70% Gly^3:Bet decreased significantly when the kale waste was first treated with EtOAc compared to the untreated biomass, probably due to poor wettability of the residual biomass. However, extraction with EtOAc after treatment with 70% Gly^3:Bet improved the yields of lutein and chlorophylls, but negatively impacted the recovery of β-carotene. It can be deduced that the prewetting of biomass with polar solvents facilitates the liberation of polar solutes like lutein and chlorophylls from their protein complexes, which in turn favours their recovery. A similar observation was reported by Mussagy et al. [65] in their work on recovering carotenoids and fatty acids from yeast. Interestingly, the integrated strategy (Route 3) achieved increased yields of both lutein (0.50 mg g^{-1} DW) and chlorophylls (7.86 mg g^{-1} DW) while maintaining

comparable yields of β-carotene (0.41 mg g^{-1} DW) and polyphenols (16.83 mg g^{-1} DW) compared to the sequential SLE processes. Moreover, using the same SLR in the extraction process, the extract from the integrated strategy was two-fold concentrated. Furthermore, the total yields of bioactive compounds obtained with this integrated process were superior to those using a conventional solvent such as methanol. The polar/non-polar properties of the mixed solvents system allowed for the concurrent solubilisation of different classes of biomolecules with different polarities. Specifically, the varying polarity of the mixed solvent system aided in the extractability of polar pigments like lutein and chlorophylls by EtOAc. In view of its synergistic effect on recovery yield, and associated advantages such as time- and energy-saving, the integrated platform seems viable for the upcycling of kale waste, as depicted in Figure 5B. Considering different applications envisioned for the two kinds of extracts, the polyphenol-rich liquid extract (with powerful antioxidant capacity) can be readily incorporated into cosmetics and food products, while the pigment-rich extract, depending on the demands of the application, needs to undergo further solvent evaporation to obtain a dry solid extract.

Kale waste upcycling can be further improved by simplifying the biomass pretreatment as lyophilisation is an energy-intensive process and best avoided in downstream processing. Hence, the feasibility of applying the SLE-LLE integrated method on the wet kale waste paste was evaluated. Since the water held within the kale waste paste could work as a diluent in the solvent system, no external water was added to the extraction process. The results (Table 2) show that the recovery yields of all bioactive compounds, except polyphenols, were greatly enhanced with the use of wet kale waste paste compared to lyophilised powder. Considering the identical efficiency of the extraction method for both types of biomass, the lower recovery yields with lyophilised kale waste powder could be due to the degradation of phytochemicals during lyophilisation-pulverisation. Similar findings were reported for bioactive compounds from raspberries after pretreatment [66]. On the other hand, there was no significant increase

in the yield of polyphenols from wet biomass, possibly due to some loss during the removal process of excess water from wet paste. In summary, the results of this work demonstrated the potential for using bio-based solvents to extract bioactive metabolites from kale waste under mild conditions. In addition, their use in the SLE-LLE integrative platform as a sustainable alternative for the efficient recovery of valuables from kale waste looks promising.

4. Conclusions

This work demonstrates the feasibility of applying NADESs to extract various bioactive metabolites, including polyphenols, carotenoids and chlorophylls, from kale waste, to develop sustainable processes for the production of natural antioxidants and pigments. In summary, hydrophilic glycerol-based NADESs resulted in polyphenol-rich extracts, whereas selective recovery of lipophilic carotenoids was exhibited by hydrophobic terpene-based NADESs. NADES based on Gly^3:Bet produced enhanced polyphenol-rich extracts under simple and mild conditions (25 °C, 30 min, SLR of 1:20 and solvent concentration of 70%). Moreover, it resulted in good stability of the extract, retaining > 90% of bioactive polyphenols, and maintained clarity within it after storing at 4 °C for 30 days. Finally, in order to address the full exploitation of kale waste, a combined approach using ternary mixtures of aqueous Gly^3:Bet and EtOAc was applied to simultaneously recover and separate bioactive compounds with different polarities into two fractions; one was a ready-to-use polyphenol-rich extract, while the other was an extract rich in carotenoids and chlorophylls.

Declaration of Competing Interest

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

Acknowledgements

This work was supported by the Singapore National Research Foundation (NRF) and the Ministry of Education under the Research Centre of Excellence Program and the NRF Competitive Research Programme (NRF-CRP21-2018-0006).

References

[1] J. Guo, K. Mao, Z. Yuan, Z. Qin, T. Xu, S.M. Bateni, Y. Zhao, C. Ye, Global food security assessment during 1961–2019, Sustainability, 13 (2021) 14005.
[2] National Environment Agency, Food Waste Management. https://www.nea.gov.sg/our-services/waste-management/3r-programmes-and-resources/food-waste-management/, 2022 (assessed 1 Nov 2022).
[3] L. Xue, G. Liu, Introduction to global food losses and food waste, in: Saving Food, Elsevier, 2019, pp. 1-31.
[4] J. Chang, M. Wang, Y. Jian, F. Zhang, J. Zhu, Q. Wang, B. Sun, Health-promoting phytochemicals and antioxidant capacity in different organs from six varieties of Chinese kale, Scientific reports, 9 (2019) 1-10.
[5] D. Šamec, B. Urlić, B. Salopek-Sondi, Kale (Brassica oleracea var. acephala) as a superfood: Review of the scientific evidence behind the statement, Critical reviews in food science and nutrition, 59 (2019) 2411-2422.
[6] K. Zhou, L. Yu, Total phenolic contents and antioxidant properties of commonly consumed vegetables grown in Colorado, LWT-Food Science and Technology, 39 (2006) 1155-1162.
[7] L.S. Huber, R. Hoffmann-Ribani, D.B. Rodriguez-Amaya, Quantitative variation in Brazilian vegetable sources of flavonols and flavones, Food Chemistry, 113 (2009) 1278-1282.
[8] E. Sikora, E. Cieślik, T. Leszczyńska, A. Filipiak-Florkiewicz, P.M. Pisulewski, The antioxidant activity of selected cruciferous vegetables subjected to aquathermal processing, Food Chemistry, 107 (2008) 55-59.
[9] L. Vargas, R. Kapoor, B. Nemzer, H. Feng, Application of different drying methods for evaluation of phytochemical content and physical properties of broccoli, kale, and spinach, LWT, 155 (2022) 112892.
[10] A. Kaulmann, M.-C. Jonville, Y.-J. Schneider, L. Hoffmann, T. Bohn, Carotenoids, polyphenols and micronutrient profiles of Brassica oleraceae and plum varieties and their contribution to measures of total antioxidant capacity, Food Chemistry, 155 (2014) 240-250.
[11] L. Ma, X.M. Lin, Effects of lutein and zeaxanthin on aspects of eye health, Journal of the Science of Food and Agriculture, 90 (2010) 2-12.
[12] C. Jubert, J. Mata, G. Bench, R. Dashwood, C. Pereira, W. Tracewell, K. Turteltaub, D. Williams, G. Bailey, Effects of chlorophyll and chlorophyllin on low-dose aflatoxin B1 pharmacokinetics in human volunteers, Cancer prevention research, 2 (2009) 1015-1022.
[13] F. Danesi, M. Govoni, L.F. D'Antuono, A. Bordoni, The molecular mechanism of the cholesterol‐lowering effect of dill and kale: The influence of the food matrix components, Electrophoresis, 37 (2016) 1805-1813.
[14] M.C. Meinke, A. Friedrich, K. Tscherch, S.F. Haag, M.E. Darvin, H. Vollert, N. Groth, J. Lademann, S. Rohn, Influence of dietary carotenoids on radical scavenging capacity of the skin and skin lipids, European Journal of Pharmaceutics and Biopharmaceutics, 84 (2013) 365-373.
[15] M. Lemos, J.R. Santin, L.C.K. Júnior, R. Niero, S.F.d. Andrade, Gastroprotective activity of hydroalcoholic extract obtained from the leaves of Brassica oleracea var. acephala DC in different animal models, Journal of Ethnopharmacology, 138 (2011) 503-507.
[16] G. Das, H.-S. Shin, J.K. Patra, Multitherapeutic Efficacy of Curly Kale Extract Fabricated Biogenic Silver Nanoparticles, International Journal of Nanomedicine, 17 (2022) 1125.

[17] Meinke, C.K. Nowbary, S. Schanzer, H. Vollert, J. Lademann, M.E. Darvin, Influences of orally taken carotenoid-rich curly kale extract on collagen I/elastin index of the skin, Nutrients, 9 (2017) 775.
[18] B. Nayak, F. Dahmoune, K. Moussi, H. Remini, S. Dairi, O. Aoun, M. Khodir, Comparison of microwave, ultrasound and accelerated-assisted solvent extraction for recovery of polyphenols from Citrus sinensis peels, Food chemistry, 187 (2015) 507-516.
[19] N.G. Meneses, S. Martins, J.A. Teixeira, S.I. Mussatto, Influence of extraction solvents on the recovery of antioxidant phenolic compounds from brewer's spent grains, Separation and purification technology, 108 (2013) 152-158.
[20] M. Borja-Martínez, J. Lozano-Sánchez, I. Borrás-Linares, M.A. Pedreño, A.B. Sabater-Jara, Revalorization of broccoli by-products for cosmetic uses using supercritical fluid extraction, Antioxidants, 9 (2020) 1195.
[21] N. Maravić, N. Teslić, D. Nikolić, I. Dimić, Z. Šereš, B. Pavlić, From agricultural waste to antioxidant-rich extracts: Green techniques in extraction of polyphenols from sugar beet leaves, Sustainable Chemistry and Pharmacy, 28 (2022) 100728.
[22] Y. Dai, E. Rozema, R. Verpoorte, Y.H. Choi, Application of natural deep eutectic solvents to the extraction of anthocyanins from Catharanthus roseus with high extractability and stability replacing conventional organic solvents, Journal of Chromatography A, 1434 (2016) 50-56.
[23] Y.H. Choi, J. van Spronsen, Y. Dai, M. Verberne, F. Hollmann, I.W.C.E. Arends, G.-J. Witkamp, R. Verpoorte, Are Natural Deep Eutectic Solvents the Missing Link in Understanding Cellular Metabolism and Physiology?, Plant Physiology, 156 (2011) 1701-1705.
[24] D.T. Silva, F.A. Smaniotto, I.F. Costa, J. Baranzelli, A. Muller, S. Somacal, C.S.A. Monteiro, M. Vizzotto, E. Rodrigues, M.T. Barcia, Natural deep eutectic solvent (NADES): A strategy to improve the bioavailability of blueberry phenolic compounds in a ready-to-use extract, Food Chemistry, 364 (2021) 130370.
[25] L. Khare, T. Karve, R. Jain, P. Dandekar, Menthol based hydrophobic deep eutectic solvent for extraction and purification of ergosterol using response surface methodology, Food Chemistry, 340 (2021) 127979.
[26] C. Fan, Y. Liu, Y. Shan, X. Cao, A priori design of new natural deep eutectic solvent for lutein recovery from microalgae, Food Chemistry, 376 (2022) 131930.
[27] W. Pitacco, C. Samorì, L. Pezzolesi, V. Gori, A. Grillo, M. Tiecco, M. Vagnoni, P. Galletti, Extraction of astaxanthin from Haematococcus pluvialis with hydrophobic deep eutectic solvents based on oleic acid, Food Chemistry, 379 (2022) 132156.
[28] A. Stupar, V. Šeregelj, B.D. Ribeiro, L. Pezo, A. Cvetanović, A. Mišan, I. Marrucho, Recovery of β-carotene from pumpkin using switchable natural deep eutectic solvents, Ultrasonics Sonochemistry, 76 (2021) 105638.
[29] Y. Jin, D. Jung, K. Li, K. Park, J. Ko, M. Yang, J. Lee, Application of deep eutectic solvents to prepare mixture extracts of three long-lived trees with maximized skin-related bioactivities, Applied Sciences, 9 (2019) 2581.
[30] B. Zheng, Y. Yuan, J. Xiang, W. Jin, J.B. Johnson, Z. Li, C. Wang, D. Luo, Green extraction of phenolic compounds from foxtail millet bran by ultrasonic-assisted deep eutectic solvent extraction: Optimization, comparison and bioactivities, Lwt, 154 (2022) 112740.
[31] C. Benoit, C. Virginie, V. Boris, The use of NADES to support innovation in the cosmetic industry, in: Advances in Botanical Research, Elsevier, 2021, pp. 309-332.
[32] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventós, [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, in: Methods in enzymology, Elsevier, 1999, pp. 152-178.

[33] N.E. Craft, J.H. Soares, Relative solubility, stability, and absorptivity of lutein and. beta.-carotene in organic solvents, Journal of agricultural and food chemistry, 40 (1992) 431-434.
[34] R. Porra, W.a.A. Thompson, P. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy, Biochimica et Biophysica Acta (BBA)-Bioenergetics, 975 (1989) 384-394.
[35] J.P. Wojeicchowski, C. Marques, L. Igarashi-Mafra, J.A. Coutinho, M.R. Mafra, Extraction of phenolic compounds from rosemary using choline chloride–based deep eutectic solvents, Separation and Purification Technology, 258 (2021) 117975.
[36] J. Cao, J. Cao, H. Wang, L. Chen, F. Cao, E. Su, Solubility improvement of phytochemicals using (natural) deep eutectic solvents and their bioactivity evaluation, Journal of Molecular Liquids, 318 (2020) 113997.
[37] F.A. Ayaz, S. Hayırlıoglu-Ayaz, S. Alpay-Karaoglu, J. Grúz, K. Valentová, J. Ulrichová, M. Strnad, Phenolic acid contents of kale (Brassica oleraceae L. var. acephala DC.) extracts and their antioxidant and antibacterial activities, Food Chemistry, 107 (2008) 19-25.
[38] J.-B. Chagnoleau, A.M. Ferreira, J.A. Coutinho, X. Fernandez, S. Azoulay, N. Papaiconomou, Sustainable extraction of antioxidants from out-of-caliber kiwifruits, Food Chemistry, (2022) 133992.
[39] M. Derrien, A. Badr, A. Gosselin, Y. Desjardins, P. Angers, Optimization of a green process for the extraction of lutein and chlorophyll from spinach by-products using response surface methodology (RSM), LWT-Food Science and Technology, 79 (2017) 170-177.
[40] W. Stahl, H. Sies, Antioxidant activity of carotenoids, Molecular Aspects of Medicine, 24 (2003) 345-351.
[41] C.-Y. Hsu, P.-Y. Chao, S.-P. Hu, C.-M. Yang, The antioxidant and free radical scavenging activities of chlorophylls and pheophytins, (2013).
[42] M.I. Gil, F.A. Tomás-Barberán, B. Hess-Pierce, A.A. Kader, Antioxidant capacities, phenolic compounds, carotenoids, and vitamin C contents of nectarine, peach, and plum cultivars from California, Journal of agricultural and food chemistry, 50 (2002) 4976-4982.
[43] R. Biegańska-Marecik, E. Radziejewska-Kubzdela, R. Marecik, Characterization of phenolics, glucosinolates and antioxidant activity of beverages based on apple juice with addition of frozen and freeze-dried curly kale leaves (Brassica oleracea L. var. acephala L.), Food Chemistry, 230 (2017) 271-280.
[44] J. Armesto, L. Gómez-Limia, J. Carballo, S. Martínez, Impact of vacuum cooking and boiling, and refrigerated storage on the quality of galega kale (Brassica oleracea var. acephala cv. Galega), LWT - Food Science and Technology, 79 (2017) 267-277.
[45] K. Goiris, K. Muylaert, I. Fraeye, I. Foubert, J. De Brabanter, L. De Cooman, Antioxidant potential of microalgae in relation to their phenolic and carotenoid content, Journal of applied phycology, 24 (2012) 1477-1486.
[46] K. Thaipong, U. Boonprakob, K. Crosby, L. Cisneros-Zevallos, D. Hawkins Byrne, Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts, Journal of Food Composition and Analysis, 19 (2006) 669-675.
[47] B. Cervantes-Paz, E.M. Yahia, J. de Jesús Ornelas-Paz, C.I. Victoria-Campos, V. Ibarra-Junquera, J.D. Pérez-Martínez, P. Escalante-Minakata, Antioxidant activity and content of chlorophylls and carotenoids in raw and heat-processed Jalapeño peppers at intermediate stages of ripening, Food Chemistry, 146 (2014) 188-196.
[48] A. Podsędek, Natural antioxidants and antioxidant capacity of Brassica vegetables: A review, LWT - Food Science and Technology, 40 (2007) 1-11.

[49] J.A. Vinson, Y.A. Dabbagh, M.M. Serry, J. Jang, Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease, Journal of Agricultural and Food Chemistry, 43 (1995) 2800-2802.
[50] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biology and Medicine, 26 (1999) 1231-1237.
[51] A.C. Kurilich, E.H. Jeffery, J.A. Juvik, M.A. Wallig, B.P. Klein, Antioxidant Capacity of Different Broccoli (Brassica oleracea) Genotypes Using the Oxygen Radical Absorbance Capacity (ORAC) Assay, Journal of Agricultural and Food Chemistry, 50 (2002) 5053-5057.
[52] M. Fiol, S. Adermann, S. Neugart, S. Rohn, C. Mügge, M. Schreiner, A. Krumbein, L.W. Kroh, Highly glycosylated and acylated flavonols isolated from kale (Brassica oleracea var. sabellica)—Structure–antioxidant activity relationship, Food Research International, 47 (2012) 80-89.
[53] U.M. Lanfer-Marquez, R.M. Barros, P. Sinnecker, Antioxidant activity of chlorophylls and their derivatives, Food research international, 38 (2005) 885-891.
[54] L. Vaillant, G. Georgescou, C. Rivollier, A. Delarue, Combined effects of glycerol and petrolatum in an emollient cream: A randomized, double‐blind, crossover study in healthy volunteers with dry skin, Journal of cosmetic dermatology, 19 (2020) 1399-1403.
[55] R. Eccles, P. Mallefet, Soothing properties of glycerol in cough syrups for acute cough due to common cold, Pharmacy, 5 (2017) 4.
[56] M. Di Gioacchino, F. Bruni, M.A. Ricci, Aqueous solution of betaine: Hydration and aggregation, Journal of Molecular Liquids, 318 (2020) 114253.
[57] T. Lafarga, I. Viñas, G. Bobo, J. Simó, I. Aguiló-Aguayo, Effect of steaming and sous vide processing on the total phenolic content, vitamin C and antioxidant potential of the genus Brassica, Innovative Food Science & Emerging Technologies, 47 (2018) 412-420.
[58] Y. Dai, J. van Spronsen, G.-J. Witkamp, R. Verpoorte, Y.H. Choi, Natural deep eutectic solvents as new potential media for green technology, Analytica chimica acta, 766 (2013) 61-68.
[59] K.M. Jeong, J. Ko, J. Zhao, Y. Jin, D.E. Yoo, S.Y. Han, J. Lee, Multi-functioning deep eutectic solvents as extraction and storage media for bioactive natural products that are readily applicable to cosmetic products, Journal of Cleaner Production, 151 (2017) 87-95.
[60] J.K.U. Ling, Y.S. Chan, J. Nandong, S.F. Chin, B.K. Ho, Formulation of choline chloride/ascorbic acid natural deep eutectic solvent: Characterization, solubilization capacity and antioxidant property, LWT, 133 (2020) 110096.
[61] V. Sant'Anna, P.D. Gurak, L.D. Ferreira Marczak, I.C. Tessaro, Tracking bioactive compounds with colour changes in foods – A review, Dyes and Pigments, 98 (2013) 601-608.
[62] A. Ahmadi, S.-A. Shahidi, R. Safari, A. Motamedzadegan, A. Ghorbani-HasanSaraei, Evaluation of stability and antibacterial properties of extracted chlorophyll from alfalfa (Medicago sativa L.), Food and Chemical Toxicology, 163 (2022) 112980.
[63] J.-H. Kang, S. Kim, B. Moon, Optimization by response surface methodology of lutein recovery from paprika leaves using accelerated solvent extraction, Food chemistry, 205 (2016) 140-145.
[64] I. Viera, A. Pérez-Gálvez, M. Roca, Green Natural Colorants, Molecules (Basel, Switzerland), 24 (2019).
[65] C.U. Mussagy, V.C. Santos-Ebinuma, K.A. Kurnia, A.C. Dias, P. Carvalho, J.A. Coutinho, J.F. Pereira, Integrative platform for the selective recovery of intracellular carotenoids and lipids from Rhodotorula glutinis CCT-2186 yeast using mixtures of bio-based solvents, Green Chemistry, 22 (2020) 8478-8494.

[66] X. Si, Q. Chen, J. Bi, X. Wu, J. Yi, L. Zhou, Z. Li, Comparison of different drying methods on the physical properties, bioactive compounds and antioxidant activity of raspberry powders, Journal of the Science of Food and Agriculture, 96 (2016) 2055-2062.

Table 1. List of NADESs studied in this work.

Type	Component 1	Component 2	Molar ratio	Acronyms	pH[a]	Viscosity at 25 °C (mPa·s)
Hydrophilic	Glycerol	Betaine	2:1	Gly^2:Bet	7.57	26.92 ± 0.52[b]
	Glycerol	Betaine	3:1	Gly^3:Bet	7.31	19.33 ± 0.35[b]
	Glycerol	D-sorbitol	2:1	Gly^2:Sor	4.54	37.54 ± 0.45[b]
	Glycerol	D-sorbitol	3:1	Gly^3:Sor	4.15	33.82 ± 0.53[b]
	Glycerol	Xylose	3:1	Gly^3:Xyl	4.11	26.29 ± 0.37[b]
	Glycerol	Glucose	3:1	Gly^3:Glu	4.71	32.41 ± 0.39[b]
	Glycerol	Fructose	3:1	Gly^3:Fru	4.43	34.65 ± 0.87[b]
	Glycerol	Urea	1:1	Gly:U	9.76	9.98 ± 0.28[b]
Hydrophobic	DL-menthol	Thymol	1:1	Men:Thy	-	36.07 ± 0.26[c]
	DL-menthol	Thymol	1:2	Men:Thy^2	-	44.26 ± 0.17[c]
	DL-menthol	Fenchyl alcohol	1:1	Men:Fen	-	54.96 ± 0.36[c]
	Thymol	Fenchyl alcohol	1:1	Thy:Fen	-	32.55 ± 0.23[c]

[a] pH of 70% hydrophilic NADES in aqueous solution.

[b] viscosity of 70% hydrophilic NADES in aqueous solution.

[c] viscosity of neat hydrophobic NADES.

Table 2. Recovery yields of total phenolics, carotenoids (β-carotene, lutein) and chlorophylls (*a* and *b*) from different forms of kale waste using the SLE-LLE integrative method.

Kale waste		Extraction method		Recovery yield (mg g^{-1} DW)				
				At bottom phase	At top phase			
Type	Water content (%)	Solvent system	SLR (g mL^{-1})	Total phenolics[a]	Carotenoids		Chlorophylls	
					β-carotene	Lutein	*a*	*b*
Lyophilised powder	None	Gly^3:Bet + EtOAc + H_2O	1:20	16.83 ± 0.30	0.41 ± 0.02	0.50 ± 0.02	6.04 ± 0.03	1.82 ± 0.02
Wet paste	74.34%	Gly^3:Bet + EtOAc	1:20	16.10 ± 0.42	0.48 ± 0.04	0.57 ± 0.05	7.83 ± 0.04	2.35 ± 0.02

[a]The total phenolics reported were in mg GAE g^{-1} DW.

Figure captions

Figure 1. Experimental setup with the main stages applied in this work.

Figure 2. (A) Recovery yields of total phenolics, carotenoids (β-carotene, lutein) and chlorophylls (*a* and *b*) from kale waste using different solvents at a SLR of 1:40 and after 30 min stirring at 150 rpm and 25 °C; **(B)** antioxidant activity measured by the DPPH assay method for the extracts obtained; and the **(C)** correlation (simple regression analysis) between antioxidant activity and total phenolics in the extracts. ND indicates not determined. Different letters in the same series indicate significant differences at $p < 0.05$ level. Solvents tested in this work included H_2O – water, MeOH – methanol, 70% MeOH – 70% aqueous methanol, EtOH – ethanol, 70% EtOH – 70% aqueous ethanol, 70% Gly – 70% aqueous glycerol, 70% aqueous NADES based on Gly^2:Bet – glycerol:betaine (2:1), Gly^3:Bet – glycerol:betaine (3:1), Gly^2:Sor – glycerol:sorbitol (2:1), Gly^3:Sor – glycerol:sorbitol (3:1), Gly^3:Xyl – glycerol:xylose (3:1), Gly^3:Glu – glycerol:glucose (3:1), Gly^3:Fru – glycerol;fructose (3:1), and Gly:U – glycerol:urea (1:1), Hex – hexane, EtOAc – ethyl acetate, Men:Thy – DL-menthol:thymol (1:1), $Men:Thy^2$ – DL-menthol:thymol (1:2), Men:Fen – DL-menthol:fenchyl alcohol (1:1) and Thy:Fen – thymol:fenchyl alcohol (1:1) (see Table 1 for more details).

Figure 3. Recovery yields of total phenolics from kale waste using the NADES based on glycerol:betaine (3:1), when investigating the effects of the **(A)** SLR, at the solvent concentration of 70% and at 25 °C for 30 min; and the **(B)** solvent concentration (50, 60, 70 and 80%), extraction temperature (25, 45 and 65 °C) and time (30 and 60 min), and in comparison with the ultrasound-assisted extraction (that was operated at 37 kHz and 100% power), using a SLR of 1:20. Different letters in the same series indicate significant differences at $p < 0.05$ level.

Figure 4. The stability of kale waste extracts produced using solvent (H_2O – water, 70% EtOH – 70% aqueous ethanol, 70% Gly – 70% aqueous glycerol and 70% Gly^3:Bet – 70% aqueous glycerol:betaine (3:1)), represented by the relative residual concentration of total phenolics over time after being stored in the dark at **(A)** 25 °C and **(B)** 4 °C, respectively, for 30 days, and the **(C)** photographs of the extracts just after extraction and during the storage. The relative residual concentration of total phenolics in the extract at the time of storage was derived by applying the control as the total phenolics in the original extract obtained with water before storage.

Figure 5. (A) Recovery yields of total phenolics, carotenoids (β-carotene, lutein) and chlorophylls (*a* and *b*) from kale waste using sequential (Route 1 and 2) and integrated (Route 3) approaches designed in this work. Different letters in the same series indicate significant differences at $p < 0.05$ level; and the **(B)** diagram of the integrative process for upcycling kale waste using green, cosmetic- and food-compatible ternary solvent mixtures (Gly^3:Bet – glycerol: betaine (3:1) + H_2O – water + EtOAc – ethyl acetate) for the production of natural antioxidants and pigments with potential applications in the cosmetic and food industries. Dashed lines were not experimentally tested but were recurrently used in product polishing.

Figure 1.

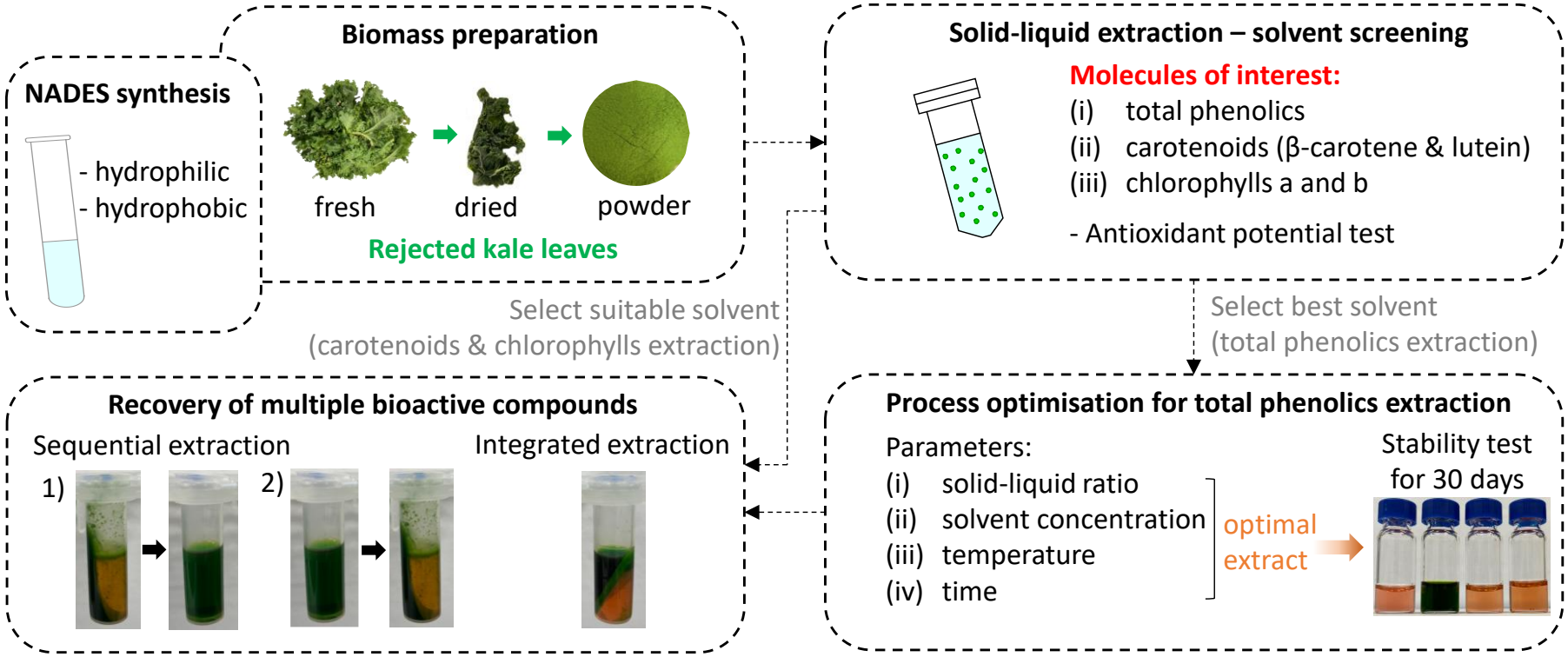

Biomass preparation
NADES synthesis
- hydrophilic
- hydrophobic
fresh
dried
powder
Rejected kale leaves
Solid-liquid extraction – solvent screening
Molecules of interest:
(i) total phenolics
(ii) carotenoids (β-carotene & lutein)
(iii) chlorophylls a and b
- Antioxidant potential test
Select suitable solvent
(carotenoids & chlorophylls extraction)
Select best solvent
(total phenolics extraction)
Recovery of multiple bioactive compounds
Sequential extraction
1)
2)
Integrated extraction
Process optimisation for total phenolics extraction
Parameters:
(i) solid-liquid ratio
(ii) solvent concentration
(iii) temperature
(iv) time
optimal extract
Stability test for 30 days

Figure 2.

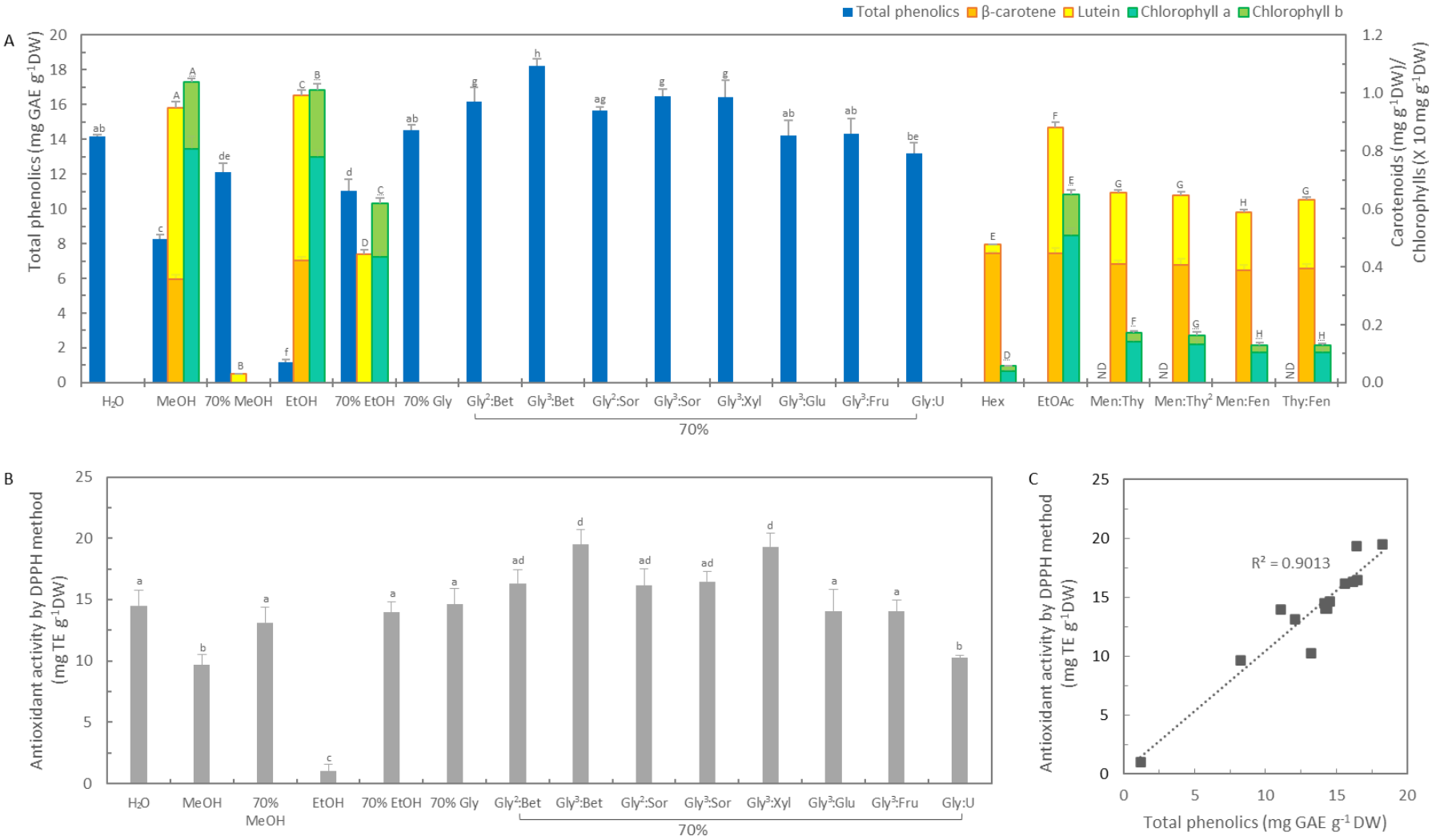

A
Total phenolics
β-carotene
Lutein
Chlorophyll a
Chlorophyll b
Total phenolics (mg GAE g-1DW)
Carotenoids (mg g-1DW)/ Chlorophylls (X 10 mg g-1DW)
H2O
MeOH
70% MeOH
EtOH
70% EtOH
70% Gly
Gly2:Bet
Gly3:Bet
Gly2:Sor
Gly3:Sor
Gly3:Xyl
Gly3:Glu
Gly3:Fru
Gly:U
Hex
EtOAc
Men:Thy
Men:Thy2
Men:Fen
Thy:Fen
70%
B
Antioxidant activity by DPPH method (mg TE g-1DW)
C
R² = 0.9013
Total phenolics (mg GAE g-1 DW)

Figure 3.

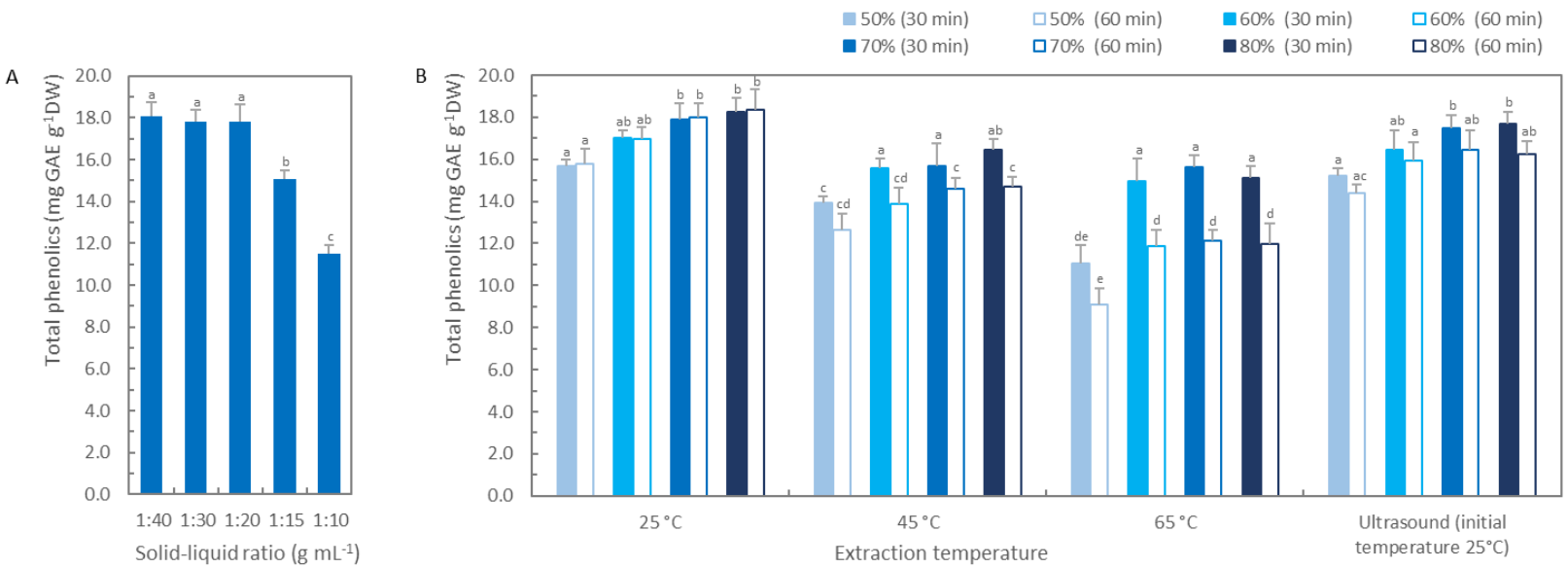

A
Total phenolics (mg GAE g-1DW)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
1:40 1:30 1:20 1:15 1:10
Solid-liquid ratio (g mL-1)
B
50% (30 min)
50% (60 min)
60% (30 min)
60% (60 min)
70% (30 min)
70% (60 min)
80% (30 min)
80% (60 min)
Total phenolics (mg GAE g-1DW)
25 °C
45 °C
65 °C
Ultrasound (initial temperature 25°C)
Extraction temperature

Figure 4.

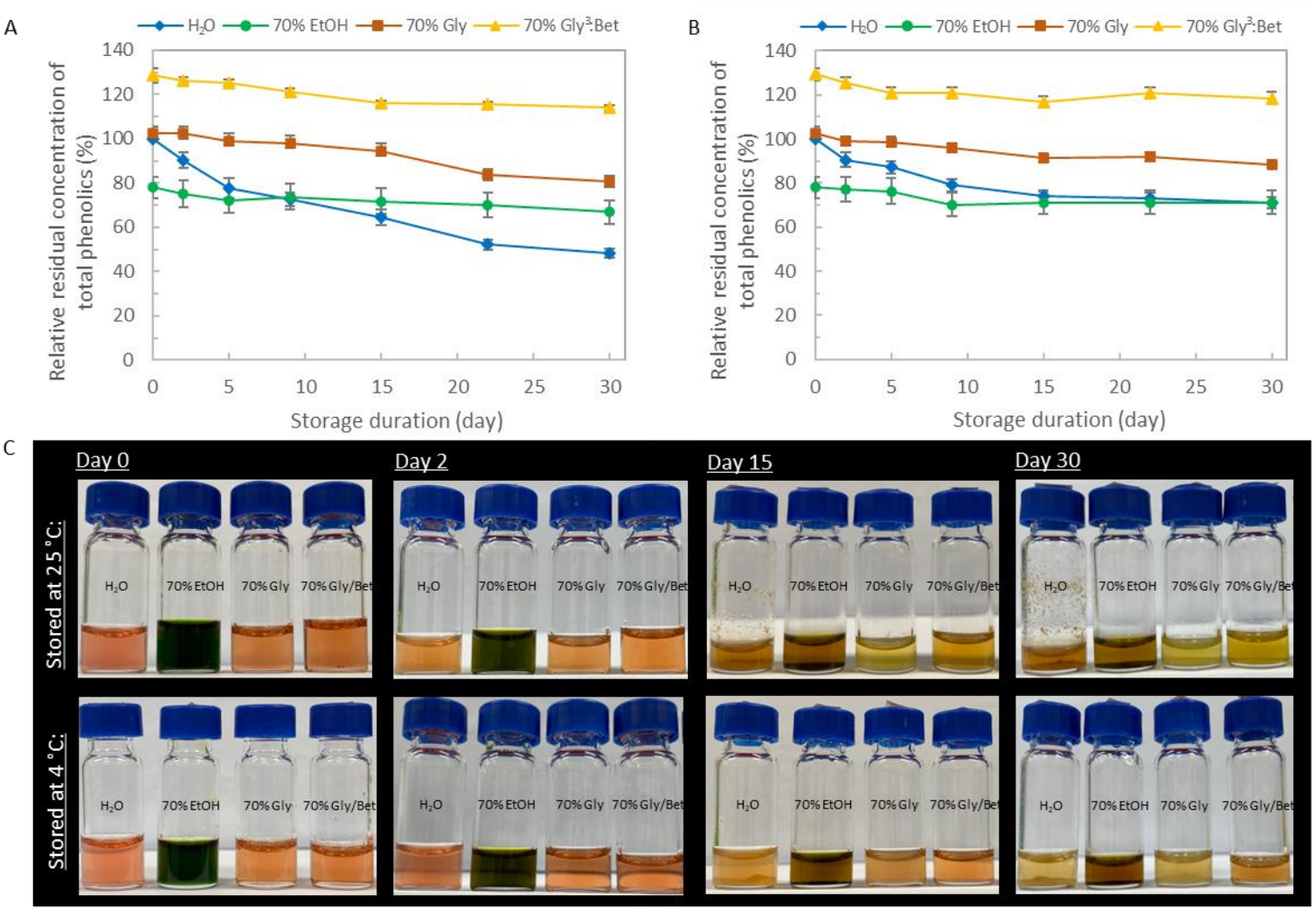
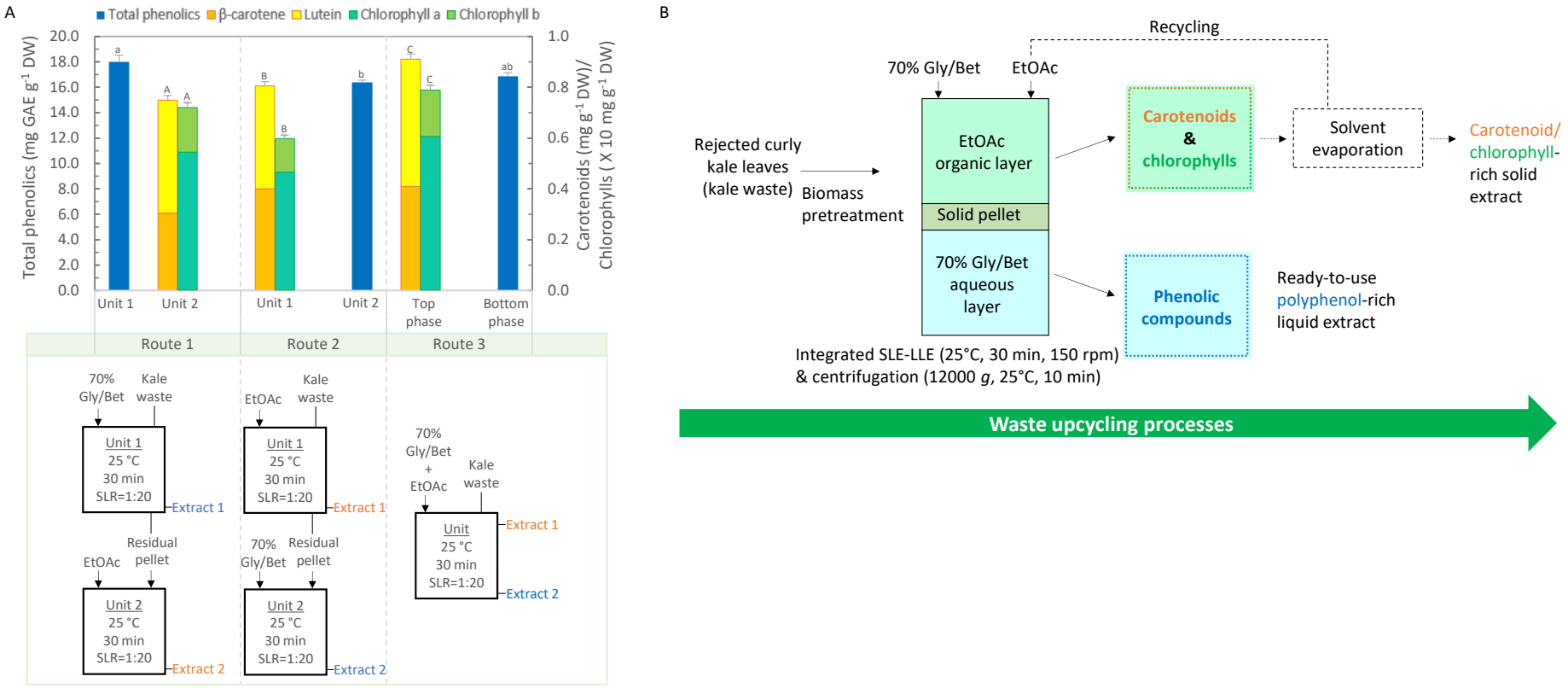

A
B
H2O
70% EtOH
70% Gly
70% Gly3:Bet
Relative residual concentration of total phenolics (%)
Storage duration (day)
C
Day 0
Day 2
Day 15
Day 30
Stored at 25 °C:
Stored at 4 °C:
H2O
70% EtOH
70% Gly
70% Gly/Bet

Figure 5.


A
Total phenolics
β-carotene
Lutein
Chlorophyll a
Chlorophyll b
Total phenolics (mg GAE g-1 DW)
Carotenoids (mg g-1 DW)/ Chlorophylls (X 10 mg g-1 DW)
Unit 1
Unit 2
Top phase
Bottom phase
Route 1
Route 2
Route 3
70% Gly/Bet
Kale waste
EtOAc
Unit 1 25 °C 30 min SLR=1:20
Extract 1
Residual pellet
Unit 2 25 °C 30 min SLR=1:20
Extract 2
Unit 25 °C 30 min SLR=1:20
B
Recycling
Rejected curly kale leaves (kale waste)
Biomass pretreatment
EtOAc organic layer
Solid pellet
70% Gly/Bet aqueous layer
Carotenoids & chlorophylls
Solvent evaporation
Carotenoid/ chlorophyll-rich solid extract
Phenolic compounds
Ready-to-use polyphenol-rich liquid extract
Integrated SLE-LLE (25°C, 30 min, 150 rpm) & centrifugation (12000 g, 25°C, 10 min)
Waste upcycling processes