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RESEARCH ARTICLE

Deep Eutectic Solvents for the Extraction and Stabilization of Radical Scavengers From Ecuadorian Quinoa (*Chenopodium quinoa* Willd.) Leaves

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Keywords: *Chenopodiaceae* | deep eutectic solvent-storage stabilization | flavonoids green extraction | natural radical quenching

ABSTRACT

Flavonoids are probable major contributors to the radical scavenging activity in Ecuadorian quinoa leaves, both from the bitter genotype (Chimborazo) and sweet varieties. In this study, we extracted these compounds using a simple, rapid (10 min), and environmentally friendly method based on deep eutectic solvents (DES). Extractions were performed in a ball mixer mill at room temperature with a eutectic mixture of choline chloride—glycerol—water at a molar ratio, of 1:2:1 and compared with classical methanol extraction. Both extracts were characterized using high-performance thin-layer chromatography and liquid chromatography-tandem mass spectrometry-based methods. Regardless of the type of solvent used (conventional or green solvent), quercetin and kaempferol glycosides were found as the major flavonols in sweet and bitter quinoa leaves. DES extract contains a higher amount of quercetin glycosides than methanol and shows a higher capacity to stabilize the quinoa radical scavengers compared to conventional solvent (liquids extracts—conservation for up to 4 months at 5°C). The present research indicates that DES represent an efficient green media for the stabilization of phenolic compounds from quinoa leaves and has the potential as a possible alternative to organic solvents. Our work opens new perspectives for the development of high-added value products based on quinoa leaves for pharmaceutical, nutraceutical and agro-food applications.

1 | Introduction

Chenopodium quinoa Willd. (*Chenopodiaceae*) leaves (harvested around 90 days of cultivation) are eaten as vegetables in some parts of Asia, and North and South America [1, 2]. Even though the leaves contain a higher content of phenolic compounds than the seeds, they are scarcely consumed and there is still limited information about their nutraceutical potential.

Polyphenols are diverse and represent a widely occurring group of phytochemicals in plants. Their major beneficial effects on health are due to their antioxidant properties and their ability to scavenge free radicals and reactive oxygen species (ROS), which are associated with their metal chelation and redox properties [3]. Amongst polyphenols, flavonoids have been extensively investigated for their possible roles in preventing degenerative diseases such as coronary heart disease, atherosclerosis, and diabetes, by antioxidative action and/or the modulation of several protein functions [4, 5]. Interestingly, an in vitro study reports the high bioavailability of quinoa leaf compounds (mainly phenolic compounds) together with their cytotoxic effect on prostate cancer cells suggesting a potential of these molecules in the prevention of cancer and ROS-related diseases [1].

A new generation of solvents, known as deep eutectic solvents (DESs), have been proposed as a promising, eco-friendly, and efficient alternative to organic solvents to extract plant metabolites. Considering the growing demand for sustainable processes in the food and pharmaceutical industries, these solvents represent a good opportunity to develop innovative extracts with unique phytochemical fingerprints and biological activities [6, 7]. DESs have recently attracted widespread scientific and industrial interests thanks to their broad range of applications [8, 9]. DESs are formed by the self-association of complementary molecular partners, namely the DES components, due to intermolecular interactions, such as van der Waals interactions, hydrogen bonding (H-bonding), and/or ionic bonding [10, 11], resulting in a mixture with a significantly lower melting point than each individual component. Most common DESs are based on a combination of a hydrogen-bond acceptor (HBA, such as choline chloride) and a hydrogen-bond donor (HBD, such as polyols, sugars, or carboxylic acids) [11, 12]. Due to their biocompatibility, non-toxicity, low vapor pressure, sustainability, environmental friendliness, and remarkable power to solubilize, DESs are proposed to replace conventional organic solvents in many chemical processes, for example, extraction, enzymatic reactions, chemical synthesis, and stabilization of natural compounds [11, 13–15]. The extraction and stabilization of high-value substances such as phenolic compounds, proteins, and saponins from natural sources using DES are now a major topic of interest. The "green challenge" is not as easy as it may appear and, until recently, the natural origin of the herbal material was enough for a botanical extract to claim to be "natural". Nowadays, expectations have become much higher and the naturalness of a commercially available plant extract must be considered as a whole: herb, farming type, eco-design, bio-based solvents, and preservatives [6].

In the present study, DES-based ball mixer mill extraction at room temperature of flavonoids from quinoa leaves of Ecuadorian sweet variety and bitter genotype is evaluated. The eutectic mixture of choline chloride: glycerol: water (molar ratio, 1:2:1) is used for the extraction of flavonoids and is compared with conventional extraction using methanol. This paper aims to investigate the potential of quinoa leaf extracts as a source of bioactive compounds, and the ability of the solvents (DES and methanol) to stabilize the radical scavengers from quinoa leaves at 5°C, in order to evaluate the potential use of DES-quinoa leaf extracts for industrial applications. Promoting the consumption of quinoa leaves offers several benefits: (i) a shorter growth cycle before harvest, with leaves reaching maturity in just 3 months, compared to 6–9 months for seeds, which helps reduce the risks associated with climate-related challenges for producers; (ii) the potential for diversifying the economic activities of quinoa farmers; and (iii) possible health advantages, as quinoa leaves may aid in the chemoprevention of cancer and other diseases linked to oxidative stress. In fact, a prior in vitro study suggested that quinoa leaf polyphenols have a high bioavailability and exhibit cytostatic and anti-invasive effects on prostate cancer cells [1].

2 | Results and Discussion

2.1 | Preparation of the DES

Choline chloride, glycerol, and water were selected as DES components at a molar ratio 1:2:1 for their advantage in terms of biodegradability [16] and their extraction power (this DES was selected from a larger study investigating eight DESs based on choline chloride, sugars, polyols, organic acids, and/or amino acids; data to be published). Glycerol as liquid HBD and water contribute to obtain a clear, stable and low viscosity DES [8, 11, 17], with reduced water activity [11]. The prepared DES was remarkably stable for long periods of time (at least 2 years at RT; Figure S2). Intermolecular forces exist between the glycosylated flavonoids and the components of the DES. It is expected that hydrogen bonds will form between the hydroxyl groups of glycerol and the glycosylated portion of the flavonoids, likely to enhance the extraction process.

2.2 | Fingerprinting and Recovery of Quinoa Flavonoids by Solid-Phase Extraction

As the coloration of the cartridges was greenish yellow after the pretreatment of quinoa leaf extracts by solid-phase extraction (SPE) (Figure S3), we calculated the recovery (Figure 1) using Equation (1). To do so, we based on the data from Table 1 which exhibits the content of phenolic acids and flavonoids (section 4.6) in methanol extracts of bitter and sweet quinoa leaves before and after the pretreatment by SPE procedure.

After the chromatographic separation of quinoa methanolic extracts, we observed yellow and green spots dominating the high-performance thin-layer chromatography (HPTLC) fingerprints of bitter and sweet quinoa leaf extracts (Figure 1), indicating abundant glycosides based on quercetin and kaempferol, respectively. These flavonols have been extensively studied, mostly in vitro, for their chemopreventive and anticarcinogenic effects [1, 18]. Interestingly Gawlik-Dziki et al. associated the presence of high quantities of flavonols kaempferol, isorhamnetin, and rutin in a quinoa leaf extract (young leaves at 90 days; ethanol 50%, v/v) with an inhibition of prostate cancer cell proliferation, motility and competence for gap junctional communication [1]. The blue spots correspond to phenolic acids [19]. The phenolic acids reported in quinoa leaves are gallic, ferulic, and sinapinic acids [1]. In a recent study, Shen et al. demonstrated that a DES composed of choline chloride and lactic acid in a 1:1 molar ratio exhibited superior extraction efficiency of chlorogenic acid

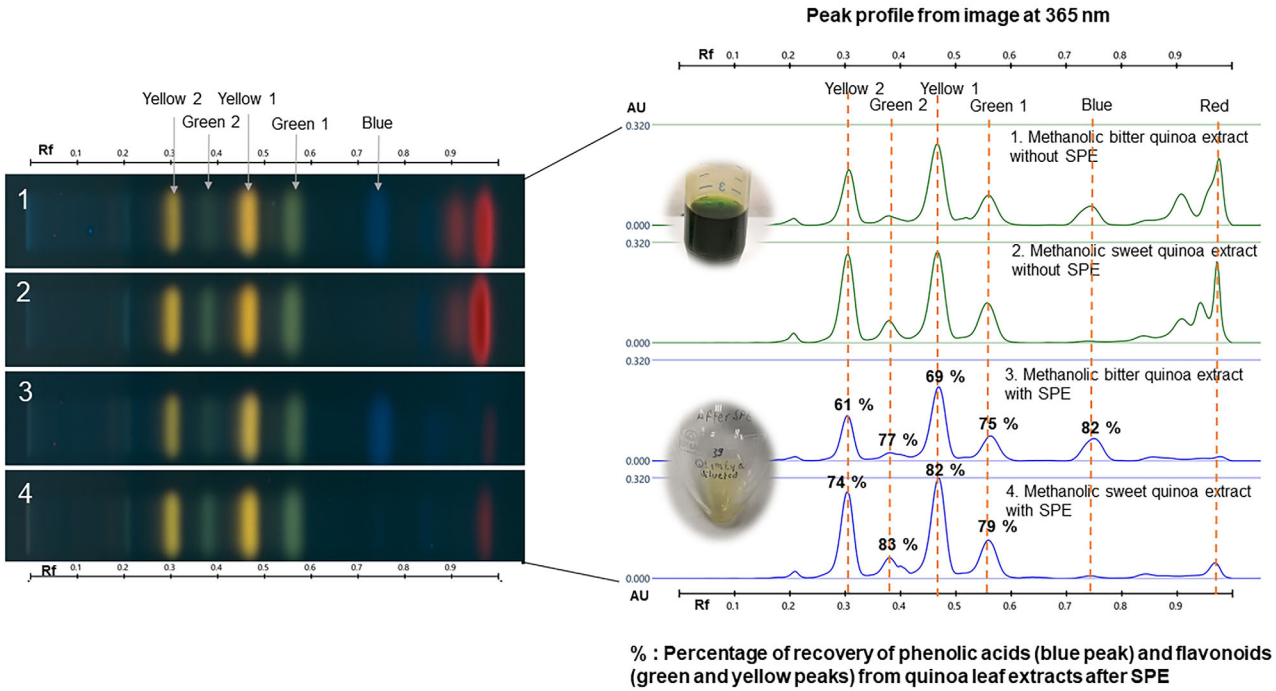

FIGURE 1 | High-performance thin-layer chromatography (HPTLC) fingerprints of flavonoids in quinoa leaf extracts (methanol; sample/solvent ratio, 1:20 w/v; application volumes, 4 μL) before (tracks 1 and 2) and after (tracks 3 and 4), solid-phase extraction treatment before application. Mobile phase: formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, v/v/v/v). Derivatization with natural product (NP) and PEG reagents; examination under UV_{365nm}. Quinoa leaves Chimborazo (tracks 1 and 3) genotype and INIAP-Tunkahuan (tracks 2 and 4) variety.

TABLE 1 | Content of phenolic acids (blue spot), and flavonoids (green and yellow spots) expressed as mg rutin-eq/g by high-performance thin-layer chromatography (HPTLC) semi-quantification of bitter and sweet quinoa leaves before and after the solid-phase extraction procedure.

		Bitter quinoa leaves			Sweet quinoa leaves		
Samples	**ID band in chromatogram**	**mg rutin-eq/g**	**SD**	**CV**	**mg rutin-eq/ g**	**SD**	**CV**
Methanolic extracts before SPE	Blue	0.99	0.07	7.05	n.d.[(a)]		
	Green 1	1.42	0.07	5.17	1.63	0.05	3.11
	Yellow 1	4.16	0.25	6.05	3.79	0.17	4.45
	Green 2	0.66	0.02	3.05	0.89	0.02	2.68
	Yellow 2	2.08	0.09	4.53	2.79	0.12	4.22
Methanolic extracts after SPE	Blue	0.81	0.10	11.95	n.d. [(a)]		
	Green 1	1.07	0.03	3.24	1.28	0.07	5.74
	Yellow 1	2.87	0.00	0.16	3.10	0.25	8.06
	Green 2	0.51	0.01	2.14	0.73	0.06	8.54
	Yellow 2	1.26	0.09	6.95	2.06	0.17	8.27

CV%: Coefficient of variation; SD: standard deviation. $N = 3$ extraction replicates.
[(a)]n.d.: Not detectable.

from Honeysuckle, compared to ethanol. They provided evidence that chlorogenic acid extracted using this DES has a significant inhibitory effect against various bacterial strains, surpassing the antibacterial activity of ethanol extracts [20].

The red spots are probably due to chlorophylls. Table 1 exhibits the results of the semi-quantification of phenolic acids (blue spots) and flavonoids (green and yellow spots).

The HPTLC fingerprints of bitter Chimborazo (Figure 1; tracks 1 and 3) and sweet INIAP-Tunkahuan (Figure 1; tracks 2 and 4) leaves show a decrease in the intensity of the main spots (blue, green, and yellow ones) after the SPE. The recoveries of the polymeric sorbent Strata-X (surface modified styrene divinylbenzene) SPE were obtained from the peak profiles of Chimborazo and INIAP-Tunkahuan quinoa leaf extracts (Figure 1), and were as follows: phenolic acids (blue peak), 82%; kaempferol glycosides

TABLE 2 | Content of phenolic acids (blue spot) and flavonoids (green and yellow spots) extracted using methanol and deep eutectic solvent (DES); the results were obtained by high-performance thin-layer chromatography (HPTLC) semi-quantification of bitter and sweet quinoa leaves and expressed as mg rutin-eq/g.

		Bitter quinoa leaves			Sweet quinoa leaves		
Samples	**ID band in chromatogram**	**mg rutin-eq/g**	**SD**	**CV (%)**	**mg rutin-eq/ g**	**SD**	**CV (%)**
Methanolic extract	Blue	0.81	0.10	11.95	n.d.[(a)]		
	Green 1	1.07	0.03	3.24	1.28	0.07	5.74
	Yellow 1	2.87	0.00	0.16	3.10	0.25	8.06
	Green 2	0.51	0.01	2.14	0.73	0.06	8.54
	Yellow 2	1.26	0.09	6.95	2.06	0.17	8.27
DES extract	Blue	0.56	0.02	2.96	n.d.[(a)]		
	Green 1	1.03	0.05	4.45	1.09	0.06	5.30
	Yellow 1	3.03	0.23	7.71	2.82	0.23	8.14
	Green 2	0.41	0.01	1.93	0.66	0.03	3.94
	Yellow 2	1.67	0.07	3.90	2.53	0.21	8.37

CV%: Coefficient of variation; SD: standard deviation. $N = 3$ extraction replicates.
[(a)]n.d.: not detectable.

(green peak 1), 75% (bitter), 79% (sweet); peak 2, 77% (bitter), 83% (sweet); and quercetin glycosides (yellow peak 1), 69% (bitter) and 82% (sweet); peak 2, 61% (bitter), and 74% (sweet).

The SPE recoveries ranged from 61% to 83%, depending on the compounds. These results are consistent with findings by Alam et al. [21] who indicated the SPE method is frequently used for the recovery of phenolic compounds from DES based on choline chloride, after extraction. However, the percentage of recovery of phenolic compounds can vary depending on the sorbent. Andrade-Eiroa et al. [22] reported recovery rates of oleacein and oleocanthal from choline chloride:xylitol and choline chloride:1,2-propanediol mixtures using a nonpolar resin XAD-16 (styrene divinylbenzene), to be 20%–33% and 67.9%–68.3%, respectively. In this sense, although the SPE can recover a wide range of organic analytes (from non-polar to very polar compounds), most of the reported sorbents and SPE protocols lack selectivity.

2.3 | HPTLC and Liquid Chromatography-Tandem Mass Spectrometry Evaluation of Quinoa Leaf Flavonoids Extractability by Methanol and DES

To compare the extractability power of DES versus methanol, we calculated the variation of phenolic acids and flavonoids between the DES and the methanol extract (mg rutin-eq/g) (Table 2: results obtained according to section 4.6, for bitter and sweet quinoa leaf extracts).

Figure 2 shows the HPTLC fingerprints of methanolic extracts (bitter and sweet; tracks 1 and 2 respectively) and DES extracts (bitter and sweet; tracks 3 and 4 respectively) pretreated by SPE before application onto to HPTLC plate. Interestingly, both solvents extracted similar types of flavonols, regardless of the variety, as corroborated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) results (Figure 3 and Table 4).

Peak profiles of bitter and sweet quinoa extracts (Figure 2) indicate: (i) a decrease of peak areas for phenolic acids (blue peak), by 31% (DES bitter quinoa extract), and kaempferol derivatives by 4% and 19% (DES bitter quinoa extract, green peaks 1 and 2 respectively), and by 15% and 11% (DES sweet quinoa extract, green peaks 1 and 2); (ii) an increase of peak areas for quercetin derivates by 6% and 33% (DES bitter quinoa extract, yellow peaks 1 and 2, respectively) and by 18% (DES sweet quinoa extract, yellow peak 2); only yellow peak 1 showed a decrease by 9% in DES sweet quinoa extract. Apparently, the DES used in this study could be selective in the extractability of certain quercetin derivatives (yellow peak 2). According to those results, even though the viscosity of DES is higher than that of methanol, and a rapid method (10 min) was used for the extraction at room temperature (~25°C), DES extracted similar or greater amounts of quercetin glycosides from both sweet and bitter leaves compared to methanol.

Our results with choline chloride:glycerol:water reflect the findings of Fraige et al. [23] who showed, on *Byrsonima verbascifolia* (L.) DC. [1] leaves, that methanol:water (7:3, v/v) and a DES, based on choline chloride:glycerol at a molar ratio 1:2 diluted by 20% (w/v) of water, both extract comparable amounts of quercetin glycosides (quercetin-*O*-hexoside, galloyl quercetin hexoside, quercetin-*O*-pentoside, and galloyl quercetin pentoside). Eutectic systems based on choline chloride with HBD consisting of polyols, carboxylic acids or amides are the most frequently reported DES for an efficient extraction of polyphenols, including flavonols [21]; the polyols are especially favored as their hydroxyl groups form abundant hydrogen bonds with polyphenols, improving their extraction [21, 23, 24]. Similarly, another DES, a mixture of L(-)-proline and levulinic acid, demonstrated high efficiency in flavonoid extraction from the flowers of *Trollius ledebouri*

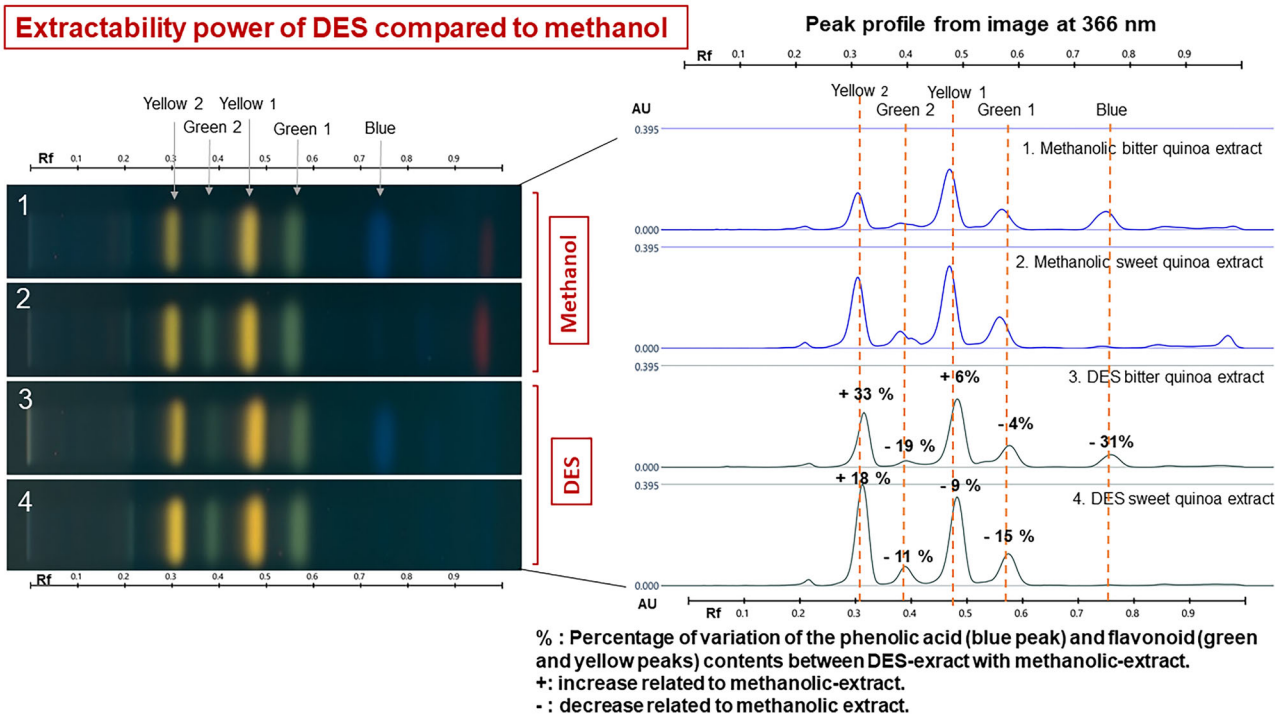

FIGURE 2 | High-performance thin-layer chromatography (HPTLC) fingerprints of flavonoids in quinoa leaf extracts (sample/solvent ratio, 1:20 w/v; application volumes, 4 μL) in methanol (tracks 1 and 2) and in deep eutectic solvent (DES) (tracks 3 and 4), treated by solid phase extraction before application. Mobile phase: formic acid–water–methyl ethyl ketone–ethyl acetate (10:10:30:50, v/v/v/v). Derivatization with natural product (NP) and PEG reagents; examination under UV_{365nm}. Quinoa leaves Chimborazo (tracks 1 and 3) genotype and INIAP-Tunkahuan (tracks 2 and 4) variety. The percentage of variation was calculated using Equation (2) in section 4.6.

Rchb.; among 20 DES synthesized, the L(-)-proline-levulinic acid combination (in a 1:2 molar ratio) exhibited the highest extraction efficiency for orientin and vitexin [25]. In another recent study on *Abelmoschus manihot* (Linn.) flowers, Wan et al. reported that the optimized DES for the extraction of hyperoside and isoquercitrin was choline chloride—acetic acid (1:2, molar ratio), while, for myricetin, the optimal eutectic mixture was choline chloride—methacrylic acid (1:2, molar ratio); the extraction of hyperoside, isoquercitrin, and myricetin with their respective optimal DES was significantly higher than that obtained using methanol, ethanol, 70% ethanol, or water [26].

In addition, phenolic acids (blue spot) were detected only in bitter quinoa leaves; their concentrations were higher in the methanol extract than in the DES extract. Furthermore, we observed that quercetin and kaempferol glycoside concentrations were slightly higher in sweet quinoa leaves than in bitter leaves, regardless of the solvent used.

On the other hand, to compare the content of flavonoids in quinoa leaves obtained from this study (DES extract and methanol extract) by HPTLC with the reported results from quinoa leaves and other natural sources, we used Equation (3) to calculate the total content of flavonoids in DES and methanol extracts from bitter and sweet quinoa leaves (Table 3); the total flavonoid content is 1.2 and 1.3 times higher for the sweet leaves over the bitter leaves, using DES and methanol, respectively. Ramos-Escudero et al. [27] found that quinoa leaves of quinoa Peruvian varieties had 8.69 (Salcedo) and 9.14 (Altiplano) mg rutin-eq/g by spectrophotometric method, which is in good agreement with the results of the present study. When comparing the flavonoid content of quinoa leaves with medicinal plants such as *Matricaria recutita* L. or *Salvia officinalis* L. *Moringa oleifera* Lam. *Spirulina platensis, Arbutus unedo* L., *Micromeria graeca* (L.) Benth. ex Rchb. *and Mentha rotundifolia* (L.) Huds, the potential of quinoa leaves as sources of bioactive compounds is remarkable (Table 3).

Figure 3 presents the mass spectra obtained when analyzing the DES and methanol extracts from quinoa leaves sweet and bitter varieties. Regarding the solvent, green or conventional, the composition of flavonoids in both extracts was similar. Tables 4 and 5 show a tentative identification of the compounds corresponding to 13 peaks as determined using LC-MS/MS analyses in the negative ionization mode from the DES and methanol extracts. Compounds were tentatively identified from experimental deprotonated molecules and their fragmentation patterns. Then, the acquired UV spectra were compared with those reported in the literature for the compounds. Peaks 2, 3, 7, and 9 at *m/z* 755, 609, 595, and 477 were tentatively assigned as quercetin glycosides presenting the fragment at *m/z* 301 characteristic of this aglycone [23] (cf. Figure S4). Peaks 1, 4, 5, 6, 10, and 12 at *m/z* 623, 739, 739, 593, 593, and 461, all showed the deprotonated aglycone as key-fragment ions at *m/z* 285, which indicates that they may have kaempferol as the aglycone [34] (Figure S5). Compounds 11 and 13 detected at *m/z* 623 and 491, respectively, produced the deprotonated aglycone fragment at *m/z* 315, which suggests that they are based on isorhamnetin [34] (Figure S5). Peak 8 at *m/z* 623 could not be assigned but the probable aglycone fragment at

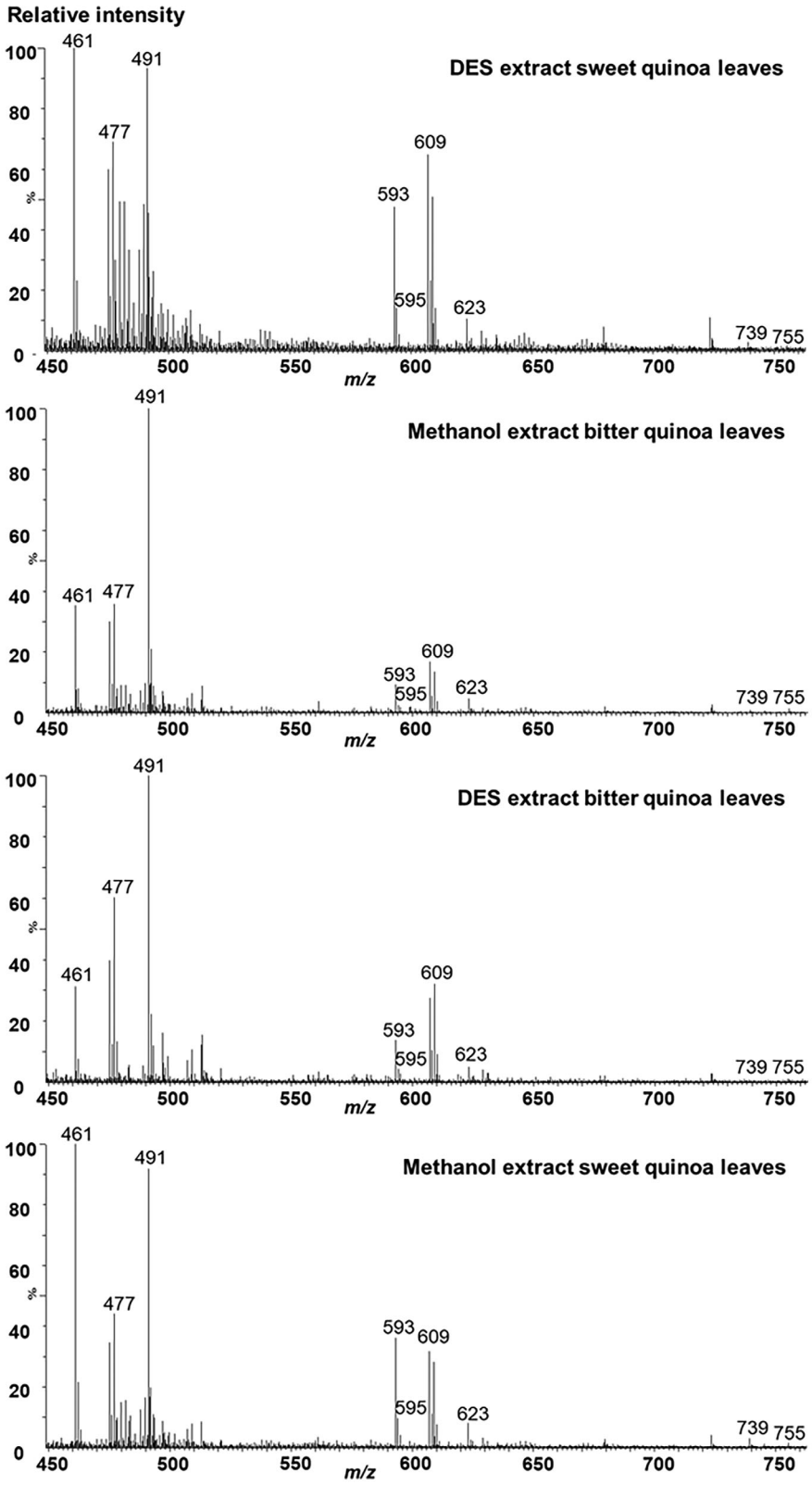

FIGURE 3 | Combined liquid chromatography-mass spectrometry (LC-MS) spectra (8–17 min run) featuring the $[M\text{-}H]^-$ ions of flavonoid glycoside from the quinoa leaf deep eutectic solvent (DES) and methanol extracts.

TABLE 3 | Comparation of total flavonoid content from studied quinoa leaves to the content of quinoa leaves and other natural sources reported in the literature.

Resources	Solvent	Method	Total flavonoids (mg rutin-eq/g)	References
Quinoa leaves, INIAP-Tunkahuan, sweet variety	DES[a]	HPTLC	7.09 ± 0.52[b]	
Quinoa leaves, Chimborazo, bitter genotype		HPTLC	6.14 ± 0.35[b]	
Quinoa leaves, INIAP-Tunkahuan, sweet variety	Methanol	HPTLC	7.18 ± 0.56[b]	
Quinoa leaves, Chimborazo, bitter genotype		HPTLC	5.70 ± 0.14[b]	
Quinoa leaves, Salcedo variety	Methanol	Spectrophotometric	8.69 ± 0.49	[27]
Quinoa leaves, Altiplano variety	Methanol	Spectrophotometric	9.14 ± 0.42	[27]
Matricaria recutita L. (synonym of *Matricaria chamomilla* L.)	Methanol	Spectrophotometric	7.1 ± 0.4	[28]
Salvia officinalis L.	Methanol	Spectrophotometric	3.5 ± 1.6	[28]
Moringa oleifera Lam. leaves	DES (bataine:urea, molar ratio 1:1)	Spectrophotometric	48–50	[29]
Moringa oleifera Lam. leaves	Ethanol 70% v/v	Spectrophotometric	20-24	[29]
Spirulina platensis	Ethanol	Spectrophotometric	0.59±0.05	[30]
Arbutus unedo L. leaves	Methanol	Spectrophotometric	21.40 ± 0.01	[31]
Micromeria graeca (L.) Benth. ex Rchb.	Ethanol	Spectrophotometric	1.90 ± 0.10[c]	[32]
Mentha rotundifolia (L.) Huds.	Ethanol 50% v/v	Spectrophotometric	3.74 ± 0.07[c]	[33]

[a]DES: extraction with DES (choline chloride:glycerol:water; molar ratios 1:2:1).
[b]Total concentration calculated using Equation (2).
[c]Total concentration reported in mg of quercetin equivalents/g of dry matter.

m/z 315 was observed, which indicates that this compound may be based on isorhamnetin.

The aglycone identification was confirmed by studying the collision-induced dissociation (CID) spectra of commercial quercetin, kaempferol, and isorhamnetin compared to the data of the natural extracts. Interestingly, the glycosyl moieties are completely lost in negative ionization electrospray. Thus, CID spectra lack information about the glycone characterization. Consequently, the CID spectra of protonated ions were also studied [35]. Indeed, those ions will undergo the fragmentation of the sugars one by one, displaying characteristic losses (-132 amu for a pentosyl unit, -146 amu for a rhamnosyl unit, -162 amu for a hexosyl unit and -176 amu for a hexuronyl unit) [35–37]. In general, the presence of pentosyl, hexosyl, and hexuronyl groups most commonly occur in the glycosyl part of flavonoids determined in quinoa leaves, regardless of solvent, DES or methanol.

About 10 flavonols, quercetin, and kaempferol glycosides have been identified in quinoa, most of them in seeds, with biological activities evaluated in vitro and in vivo such as antibacterial, antioxidant, anti-inflammatory, antitumor, anticancer, hepatoprotective, antiulcerogenic, antidiabetic activities and neuroprotective effect [18]. There is little information on the identification of flavonoids in quinoa leaves and the presence of isorhamnetin, kaempferol, quercetin and rutin has been reported [1, 18].

2.4 | Evaluation of the Stability of the Extracts Free Radical Scavenging Activity

Compared to the traditional solvents used to extract free radical scavengers from quinoa sweet and bitter leaves, DES better stabilizes this activity in liquid extracts stored at 5°C for up to 4 months (Figure 4), probably through an increase in viscosity and a reduction in water activity. All the extracts were individually evaluated, indicating an influence of storage time on free radical scavenging activity. Interestingly, both the extracts of bitter and sweet quinoa leaves in DES presented (i) a higher free radical scavenging activity compared to methanol extracts; this result is in agreement with Caprin et al. [6], who showed, for a *Calendula officinalis* L. DES extract, a dose-related radical scavenging activity significantly higher compared to an ethanol/water, 50:50 v/v extract; and (ii) a lower% of degradation in comparison to those measured in methanol.

Our data support the stabilizing effects previously reported for redox-sensitive compounds. At 5°C, DES based on choline chloride, glycerol, and water (molar ratio 1:2:1) stabilized up to 2 months saponins from bitter seeds and husks of quinoa (*Chenopodium quinoa* Willd.) [38]. At 25°C, four DES (based on the HBA choline chloride and the HBD glycerol, lactic acid, 1,2-propanodiol or oxalic acid, added with 10% water) showed a high capacity to stabilize polyphenols of rosemary (*Rosmarinus officinalis* L.) up to 72 h [39]. At 25, 4, and -20°C, DES based on choline chloride and lactic acid, added with 20% water, stabilized

TABLE 4 | Determination of tentative flavonoids in methanol- and deep eutectic solvent (DES)-extracts from *Chenopodium quinoa* Willd. leaves by mass spectrometry.

N° peak	Flavonoids	Molecular formula	*m/z* $[M-H]^-$	Δ (ppm)[(a)]	Retention time (min)	Leaves of bitter quinoa		Leaves of sweet quinoa	
						Methanolic extract[(b)]	DES extract[(c)]	Methanolic extract[(b)]	DES extract[(c)]
1	Kaempferol-O-hexuronylhexoside	$C_{27}H_{28}O_{17}$	623.1248	0.3	8.23	√	√	√	√
2	Quercetin-O-hexosyldirhamnoside	$C_{33}H_{40}O_{20}$	755.2035	0.4	9.91	√	√	√	√
3	Quercetin-O-hexosylpentoside	$C_{26}H_{28}O_{16}$	595.1299	1.3	10.80	√	√	√	√
4	Kaempferol-O-hexosyldirhamnoside–n°1	$C_{33}H_{40}O_{19}$	739.2086	1.2	11.09	√	√	√	√
5	Kaempferol-O-hexosyldirhamnoside–n°2	$C_{33}H_{40}O_{19}$	739.2086	1.2	11.36	√	√	√	√
6	Kaempferol-O-hexosylrhamnoside	$C_{27}H_{30}O_{15}$	593.1506	0.5	11.75	√	√	√	√
7	Quercetin-O-hexuronylpentoside	$C_{26}H_{26}O_{17}$	609.1092	0.5	11.85	√	√	√	√
8	Unknown flavonoid	$C_{26}H_{24}O_{18}$	623.0884	1.4	11.85	√	√	√	√
9	Quercetin-O-hexuronide	$C_{21}H_{18}O_{13}$	477.0669	0.0	13.42	√	√	√	√
10	Kaempferol-O-hexuronylpentoside	$C_{26}H_{26}O_{16}$	593.1143	1.0	13.85	√	√	√	√
11	Isorhamnetin-O-hexuronylpentoside	$C_{27}H_{28}O_{17}$	623.1248	0.8	14.20	√	√	√	√
12	Kaempferol-O-hexuronide	$C_{21}H_{18}O_{12}$	461.0720	0.7	15.57	√	√	√	√
13	Isorhamnetin-O-hexuronide	$C_{22}H_{20}O_{13}$	491.0861	0.4	16.15	√	√	√	√

[(a)]Δ (ppm): mass measurement error in parts per million (ppm).
[(b)]Methanolic extract = extraction with methanol.
[(c)]DES extract = extraction with DES (choline chloride—glycerol—water; molar ratios 1:2:1).

anthocyanins for up to 90 days [40]. Other DES stabilizing effects have been shown for *Carthamus tinctorius* L. carthamin (a C-glucosyl quinochalcone), *Catharanthus roseus* (L.) G.Don anthocyanins and *Camellia sinensis* (L.) Kuntze catechins [41]. The stabilizing ability of DES may be tentatively explained by intermolecular interactions, mainly hydrogen bonding, between solutes and DES. These interactions (i) reduce the mobility of the solutes, thus reducing their contact with oxygen and water, consequently reducing their oxidative and hydrolytic degradation; and (ii) reduce the water activity. Some authors postulate that the hydrogen bond network formed in the DES system (DES components, water, and solutes) may contribute to building up and maintaining a 3D structure of the bioactive compounds that protects them from external factors [39, 41].

These data on DES extraction of bioactive components are encouraging for further studies regarding efficiency, stability of bioactive compounds in the finished products, production costs, greenness, and potential effects on human health and safety so that these methods can be implemented by food and pharmaceutical industries [42].

Due to their radical scavenging properties, polyphenols such as flavonoids and phenolic acids are considered major contributors to the antioxidant activity of quinoa leaves [1]. Taking into account that quercetin glycosides contains two hydroxyl groups in the position 3' and 4' of the B-ring (flavonoid structure have 3 rings A, B, and C) in comparison with one hydroxyl group in the position 4' of B-ring of kaempferol glycoside; and that

TABLE 5 | Tandem mass spectrometry (MS/MS) product ions obtained from the $[M+H]^+$ and $[M-H]^-$ ions corresponding to peaks 1–13 from quinoa leaves extract, regardless of the type of extraction solvent used.

Peak number	Positive mode			Negative mode		
	$[M+H]^+$ *m/z*	CE (eV)	Fragment *m/z* (% intensity)	$[M-H]^-$ *m/z*	CE (eV)	Fragment *m/z* (% intensity)
1	625.1405	5	625 (100), 449 (10), 287 (5)	623.1248	15	623 (100), 461 (10), 447 (17), 285 (17)
					45	301 (20), 300 (100), 271 (17), 255 (7), 175 (3), 151 (2)
2	757.2191	5	757 (100), 611 (12), 465 (6), 303 (14)	755.2035	30	755 (100), 301 (11), 300 (29)
					45	301 (20), 300 (100), 271 (17), 255 (7), 175 (3), 151 (2)
3	597.1456	5	597 (100), 465 (12), 303 (6)	595.1299	30	595 (23), 301 (25), 300 (100), 271 (31)
					45	301 (19), 300 (100), 271 (76), 255 (34), 243 (8), 227 (3), 151 (4)
4	741.2242	5	741 (100), 595 (16), 449 (10), 287 (4)	739.2086	30	739 (100), 285 (17), 284 (30)
					45	285 (32), 284 (100), 255 (26), 227 (6)
5	741.2242	5	741 (100), 595 (25), 449 (13), 287 (5)	739.2086	30	739 (100), 285 (16), 284 (37)
					45	285 (30), 284 (100), 255 (28)
6	595.1663	5	595 (100), 449 (20), 433 (2), 287 (20)	593.1506	30	593 (18), 285 (42), 284 (100), 255 (6)
					45	285 (30), 284 (100), 255 (85), 227 (34), 175 (9)
7	611.1248	5	611 (100), 479 (51), 435 (1), 303 (10)	609.1092	15	609 (100), 301 (28), 300 (28)
					45	301 (100), 300 (38), 283 (10), 271 (55), 255 (31), 243 (16), 227 (10), 179 (29), 151 (59)
8	–	–	–	623.0884	30	623 (9), 315 (100), 299 (80), 287 (49), 273 (36), 271 (27), 179 (14), 151 (11)
9	479.0826	–	–	477.0669	15	477 (61), 301 (100), 300 (41)
					30	301 (100), 300 (24), 283 (5), 271 (19), 255 (7), 179 (14), 151 (23)
10	595.1663	5	595 (100), 463 (60), 287 (12)	593.1143	15	593 (100), 307 (9), 285 (61)
					45	285 (100), 257 (25), 241 (6), 229 (37), 151 (3)
11	625.1405	5	625 (100), 493 (48), 317 (8)	623.1248	15	623 (100), 315 (64), 307 (7)
					45	315 (50), 300 (100), 271 (42), 255 (24), 243 (10)
12	463.0876	–	–	461.072	15	461 (27), 285 (100)
					45	285 (100), 257 (23), 255 (20), 229 (94), 211 (24), 187 (39), 159 (20), 143 (16)
13	493.0982	–	–	491.0861	15	491 (22), 315 (100), 300 (3)
					45	315 (3), 300 (54), 255 (44), 243 (31), 227 (7), 199 (3), 151 (4)

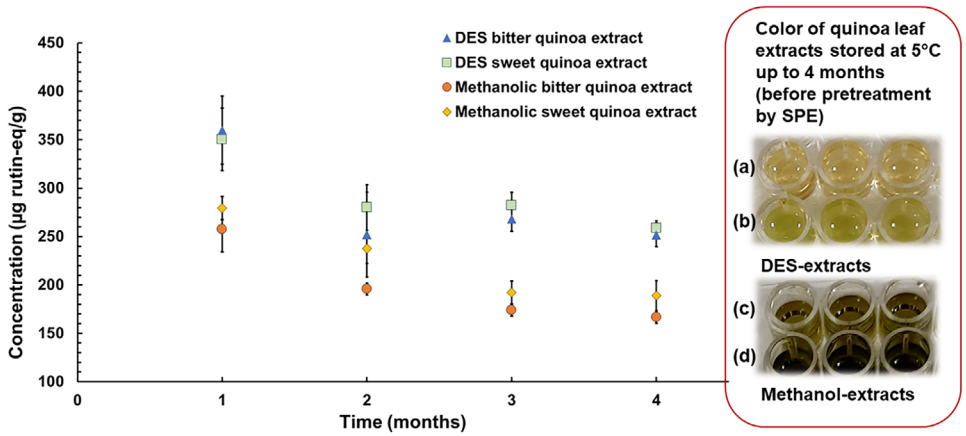

FIGURE 4 | Influence of time on a decrease in radical scavenging from quinoa leaf samples, in deep eutectic solvent (DES) (choline chloride:glycerol:water at molar ratio 1:2:1) and methanol at 5°C. Values are expressed as means of three extraction replicates with the error bars corresponding to standard deviations. (a) and (b) DES bitter and sweet quinoa leaf extracts, respectively. (c) and (d) Methanolic bitter quinoa leaf extracts, respectively.

the free radical scavenging capacity is primarily attributed to the high reactivities of the B-ring hydroxyl substituents [43], in our case, the higher content of quercetin glycosides (Table 2 and Figure 2) in DES extracts may contribute to higher 2,2-diphenyl-1-picrylhydrazyl assay (DPPH$^{\bullet}$) scavenging compared to methanol extract.

Another remarkable advantage of DES in extracting compounds from quinoa leaves was the color of its extracts which was conserved for up to 4 months. Methanol extracts presented some small sedimented particles at the bottom of the glass containers after one month, and the color darkened and became less green after 4 months (Figure 4).

3 | Conclusion

A green and rapid extraction method using DES-based ball mixer mill extraction at room temperature of flavonoids from Ecuadorian quinoa leaves, bitter (Chimborazo genotype), and sweet (INIAP-Tunkahuan variety) was evaluated. In this study, we refer to a green method primarily due to the avoidance of organic solvents, which is a key aspect. This approach also has a positive impact on the extraction process (the left-over material contains no toxic residual solvent; the DES extract is used as is, without the energy required for drying or solvent waste). However, the 'greenness' of a method indeed depends on many parameters out of our reach, notably the production process of DES components. A DES prepared with choline chloride, glycerol, and water at a molar ratio of 1:2:1 was compared to methanol, using HPTLC and LC-MS/MS methods to determine the qualitative flavonoid composition of the extracts. Regardless of the type of solvent used (conventional or green solvent), quercetin, kaempferol, and isorhamnetin glycosides were found as the major flavonols in sweet and bitter quinoa leaves. Those compounds were recovered by SPE at recovery rates oscillating between 61% and 83%. DES showed higher specificity toward quercetin glycosides than methanol, and a higher capacity to stabilize quinoa radical scavengers. These findings indicate that DESs have huge potential in industrial application due to their advantages over conventional solvents such as the possibility to design them for specific purposes, with reasonable stability of the compounds during storage. Although, it is necessary to continue investigating the use of DES in the extraction of compounds from natural sources, they are a promising alternative.

4 | Methods

4.1 | Plant Material

Sweet quinoa leaves INIAP-Tunkahuan variety were grown at the *Instituto Nacional de Investigaciones Agropecuarias* (INIAP), Santa Catalina Experimental Station, Pichincha province, 0°22'01''S 78°33'17''W, altitude 3050 m.a.s.l., and bitter organic quinoa leaves Chimborazo genotype were obtained from several producers in Calpi, Chimborazo province, 1°38'48''S 78°43'47''W, altitude 3060 m.a.s.l., both places located in Ecuador. After 90 days (sweet variety) and 75–90 days (bitter genotype) of seeding, leaves were collected manually and washed with distilled water. After removing the excess water with a paper towel, sweet quinoa leaves were lyophilized (1 kg of fresh leaves), and bitter quinoa leaves (18 kg of fresh leaves) were dried in an oven (24 h, 30°C); then both were ground and stored at -20°C. All material was ground in a laboratory mill (PX-MFC 90 D; Kinematica AG, Switzerland) and sieved to obtain particle sizes ≤0.5 mm.

4.2 | Reagents and Standards

Choline chloride (≥98%), rutin hydrate (≥94%), 2-aminoethyl diphenylborinate (97%), DPPH$^{\bullet}$, and kaempferol (>90%) were purchased from Sigma (Merck); glycerol (99%), polyethyleneglycol 400 (PEG-400), methanol with 0.1% ammonium acetate (LC-MS grade) and formic acid (99-100%) were obtained from ChemLab; methanol (99%), absolute ethanol (≥99.8%), methyl ethyl ketone (GPR Reactapur), formic acid (98%), ethyl acetate (ACS reagent), and methanol (99,8%) were purchased from VWR Chemicals; isorhamnetin (>99%) was obtained from Carl-Roth; and quercetin dihydrate (82%, Ph. Eur. Ref. Std.) from

EDQM (European Directorate for the Quality of Medicines & HealthCare). All reagents were of analytical grade. The water used to prepare DES was Milli-Q grade water (18.0 MΩ.cm). SPE cartridges, Strata -X 33 μm, Polymeric Sorbent, were purchased from Phenomenex.

4.3 | Preparation of DES

A reported eutectic mixture based on choline chloride, glycerol, and water at a molar ratio of 1:2:1 was prepared using a heating method [38]. The components were placed in a glass-closed system with magnetic stirring (350 rpm, MR Hei-Tec magnetic stirrer) at 50°C for 20 min; a water bath was used to heat the system. A viscous and transparent liquid mixture was obtained.

4.4 | Preparation of Extracts

A 0.150 g of quinoa leaf powder was mixed with 3.000 g of solvent, DES (choline chloride:glycerol:water at a molar ratio 1:2:1) or methanol, added with 9.4 mg of glass beads or 5 units (diameter, 1–1.5 mm) and extracted using a ball mixer mill MM 400 (Retsch, Germany) at 30 Hz for 10 min at room temperature (~25°C). After centrifugation (40 min, 4000 g, 25°C) (centrifuge UniCen MR, Herolab, Germany), the supernatant was recovered and stored in an amber glass 5mL-vial with a screw cap. Methanol extracts and DES extracts from bitter and sweet quinoa leaves were obtained in triplicate.

4.4.1 | Pretreatment of Extracts by SPE

Although DES extracts from quinoa leaves have the potential to be used at an industrial scale without the need to separate the extracts from DES components, we performed SPE for analytical purposes to avoid: (i) components of DES that interfere with the HPTLC chromatographic separation [44]; and (ii) the chlorophyll color in the methanolic samples, which interfere in the spectrophotometry determination in the DPPH$^{\bullet}$ assay. Prior to SPE, methanolic extracts and DES extracts were diluted as follows: (a) 0.500 g of DES extract was diluted adding 0.500 g of DES solvent and vortexed for 4 min at 28 rpm; (b) for solvent exchange, 0.500 g of methanol extract were added to 1.000 g of DES solvent previously weighed in an Eppendorf-5 mL; this mixture was carefully vortexed for 2 min at 14 rpm and methanol was then evaporated in a sample centrifugal vacuum concentrator (40°C, vacuum 12 mbar, 140 min; SP Genevac miVac SpeedTrap) to obtain the extract in 1 g DES. Both final mixtures from (a) and (b) were subsequently subjected to the SPE process. A cartridge (Strata -X 33 μm, reverse phase for neutral compounds, 30 mg/1 mL polymer-based sorbent mass) was placed in a vacuum manifold and equilibrated with 5 mL of absolute ethanol, followed by 5 mL of Milli-Q grade water. After loading 1 g of diluted extract, the cartridge was rinsed twice with 6 mL of milliQ water and eluted with 6 mL of absolute ethanol. After evaporation of ethanol at 40°C in rotavapor, the solid residue was dissolved in 1 mL of methanol to obtain SPE pretreated DES or methanol extract that was subjected to HPTLC and DPPH$^{\bullet}$ assays.

4.5 | Characterization of Extracts

4.5.1 | Liquid Chromatography-MS/MS

Instead of SPE pretreatment, the sweet and bitter quinoa leaf extracts were simply diluted for LC-MS/MS analysis of the flavonoids. The system consisted of a Waters Acquity UPLC H-class liquid chromatography device coupled to a Waters Synapt G2-S*i* mass spectrometer. Samples for injection were prepared by diluting 10 μL of sample (DES or methanolic quinoa extracts) with 990 μL methanol. Then 5 μL of each mixture was injected into the system and chromatographic separation was achieved on a non-polar column (Phenomenex Kinetex C18 EVO (00F-4633-AN), 150 × 2.1 mm; 5 μm particle size) at 40°C. The mobile phase consisted of 0.1% formic acid in water (A) and methanol (B) at a flow rate of 0.25 mL/min in gradient elution mode (0 min, 10% B; 6 min, 30% B; 11 min, 35% B; 18 min, 50% B; 23 min, 90% B; 25 min, 100% B; 27 min, 10% B). The mass spectrometer parameters were: electrospray ionization in the negative ionization mode (ESI-); dry nitrogen (ESI gas) at 500 L/h for the desolvation gas; capillary voltage 2.5 kV; cone voltage 40 V; source Offset 80 V; source temperature 120°C; desolvation temperature 300°C. Ion monitoring mode was a full scan in the range *m/z* 50–2000 and all ions were transmitted into the pusher region of the time-of-flight analyzer for mass analysis with 1 s integration time. For the analysis in the positive ionization mode, the mass spectrometer used was the Waters QToF API-US (ESI) coupled to the Waters Alliance 2695 HPLC system. All the parameters were the same as in the negative ionization mode, except for the capillary voltage which was 3.1 kV, and the cone voltage which was 30 V. Data were processed through the MassLynx V 4.1 software (Waters).

4.5.2 | High-performance TLC

Fingerprinting of flavonoid compounds contained in the extracts obtained in section 4.4.1 was performed by HPTLC according to the method reported by Liu et al. [44] and following the procedures of the European Pharmacopeia 10 [45] with some modifications. Chromatographic layers were HPTLC silica gel 60 F_{254} plates, size 20×10 cm (Merck, Germany). Samples were applied by spray, using an Automatic TLC Sampler (ATS 4, Camag, Switzerland); 5 μL of the sample were applied onto the plate for the flavonoid detection protocol; 19 bands of 5-mm were applied per plate, 8 mm from the plate lower edge; the plates were equilibrated under a 33% relative humidity and developed over a pathway of 70 mm from the lower edge in an Automated Multiple Development chamber (AMD2, Camag) with formic acid—water—methyl ethyl ketone—ethyl acetate (10:10:30:50, v/v/v/v) in a saturated twin trough chamber. The plate was automatically dried for 5 min after development and heated at 105°C for 5 min using a TLC Plate Heater (Camag). Post-chromatographic derivatization was achieved following these steps: (i) a 2 mL solution of 2-aminoethyl diphenylborinate (10 g/L in methanol; "Natural Product" reagent, NP) were applied on the warm plate (Derivatizer Camag, green nozzle, level 3); the plate was then dried for 30 s at RT; and (ii) a 2 mL solution polyethyleneglycol 400 (50 g/L in methanol; "PEG reagent") was applied on the same plate (Derivatizer Camag, blue nozzle, level 2); the plate was dried for 90 s at RT. Upon derivatization, the

plates were documented as digital images under short-wave UV light (254 nm), long-wave UV light (365 nm), and white light using the TLC Visualizer 2. The Camag systems were driven by the visionCATS software, version 2.5.

4.6 | Semi-quantification of Quinoa Leaf Flavonoids by HPTLC

To construct a calibration curve, several solutions of rutin hydrate (>94%) (20, 40, 60, 80, 160, and 200 µg/mL) were applied (4 µL) in duplicate on an HPTLC plate. DES extracts or methanolic extracts were applied in triplicate on the same plate. The plate was developed, derivatized, and recorded as described in section 4.5.2. Using CAMAG Visualizer 2, an electronic image was captured of the chromatogram in long-wave UV (366 nm, broadband) after derivatization. For each Rf value, the mean luminance of the pixels (red, blue, and green) in the zone corresponding to an aliquot of the track (50% of its length) is calculated. Luminance as a function of the Rf plot generates the "*Peak Profile from Image*" (PPI) with visionCATS software. The calibration curve was constructed by plotting peak area (AU) as a function of the rutin concentration (µg/mL) using the winCATS software.

The phenolic acid and flavonoid contents were expressed in mg rutin-eq/g (mg rutin-equivalents per gram of dried quinoa leaves). The percentage of variation of content (mg rutin-eq/g) in DES extract related to content (mg rutin-eq/g) in methanolic extract, was calculated using Equation (1).

$$\%Variation = \frac{C_{DES} - C_{MetOH}}{C_{MetOH}} \times 100 \quad (1)$$

where C_{DES} = phenolic acid or flavonoid content of DES extract (mg rutin-eq/g), and C_{MetOH} = phenolic acid or flavonoid content of methanol extract (mg rutin-eq/g).

The total flavonoid content of DES and methanolic extract (section 4.4.1) was calculated using Equation (2).

$$\text{Total flavonoid content} = C_{\text{green }1} + C_{\text{yellow }1} + \cdots + C_{\text{n-green}} + C_{\text{n-yellow}} \quad (2)$$

where $C_{\text{green }1}$ = flavonoid content (mg rutin-eq/g) in green band 1 of HPTLC chromatogram; $C_{\text{yellow }1}$ = flavonoid content (mg rutin-eq/g) in yellow band 1 of HPTLC chromatogram; $C_{\text{n-green}}$ = flavonoid content (mg rutin-eq/g) in *n*-th green band of HPTLC chromatogram; and $C_{\text{n-yellow}}$ = flavonoid content (mg rutin-eq/g) in *n*-th yellow band of HPTLC chromatogram.

4.7 | SPE: Recovery of Phenolic Compounds

We employed the methanol extracts obtained in sections 4.1 and 4.1.1 to calculate the recovery of phenolic acids and flavonoids using the SPE pretreatment by Equation (3).

$$\%\ \text{Recovery} = \frac{\text{Concentration after SPE}}{\text{Concentration before SPE}} \times 100 \quad (3)$$

where Concentration after SPE = concentration of phenolic acids (blue spot in HPTLC chromatogram) or flavonoids (green and yellow spots in HPTLC chromatogram) in mg rutin-eq/g after the SPE; and Concentration before SPE = concentration of phenolic acids (blue spot) or flavonoids (green and yellow spots) in mg rutin-eq/g without SPE pretreatment.

4.8 | Measurement of Extracts Free Radical Scavenging by a DPPH●

The radical quenching activity of DES and methanolic extracts of sweet and bitter quinoa leaf samples were measured using a DPPH● spectrophotometric assay employing a 96-well microplate, as previously reported [46]. The samples were diluted to appropriate concentrations such that they were within the dynamic range of the calibration curve (4.0, 8.0, 12.0, 16.0, 20.0, and 24.0 µg/mL of rutin). Fresh DPPH● solution (0.004% w/v in methanol with 0.1% ammonium acetate) was prepared the same day as the measurement and stored at 5°C until use. Figure S1 shows the composition, distribution, and applied volume (µl) of blanks, DPPH control, calibration standard solutions, and samples in the 96-well microplate. Absorbances were recorded at 517 nm (SpectraMax M2 microplate reader, Molecular Devices) at regular intervals of 5 min, for 40 min, 9 reads, shaking 10 s before first read and 3 s between reads. Calculation of scavenging effects relative to the control was performed per Equation (4).

$$\text{DPPH scavenging effect (\%)} = \frac{A_{\text{DPPH control cor}} - A_{\text{sample cor}}}{A_{\text{DPPH control cor}}} \quad (4)$$

where $A_{\text{DPPH control cor}}$ = Average of corrected DPPH control absorbance (average of negative control absorbances—average of blank for DPPH control absorbances); $A_{\text{sample cor}}$ = corrected absorbance of the sample (absorbance of the sample–absorbance of sample blank). All information about blanks, DPPH control, and samples is presented in Figure S2.

4.9 | Study of Free Radical Scavenger Stability of the DES and Methanol Extracts Prepared in Section 4.4

The DES and methanol extracts are prepared in section 4.4. were stored at 5°C for 4 months and regularly analyzed by DPPH● assay for their free radical scavenging activity according to the method described in section 4.7.

Author Contributions

Amandine Nachtergael: Conceptualization, funding acquisition, supervision, validation, and writing - review and editing. **Verónica Taco**: Conceptualization, methodology, investigation, formal analysis, visualization, and writing–review & editing. **Irène Semay**: Methodology, investigation, formal analysis, visualization, and writing–review & editing. **Elena Villacrés**: Investigation, analysis, and writing–review & editing. **Javier Santamaría**: Resources and writing–review & editing. **Ronny Flores**: Resources and writing–review & editing. **Pascal Gerbaux**: Guidance, resources, and writing–review & editing. **Pierre Duez**: Conceptualization, supervision, guidance, validation, and writing–review & editing. **Amandine Nachtergael**: Conceptualization, supervision, guidance, validation, and writing–review & editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.