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Extraction of Antioxidant Phenolics from Agri-Food Waste Biomass Using a Newly Designed Glycerol-Based Natural Low-Transition Temperature Mixture: A Comparison with Conventional Eco-Friendly Solvents

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Abstract: A novel natural low transition temperature mixture (LTTM), composed of glycerol and ammonium acetate (molar ratio 3:1), was tested for its efficacy as a solvent in recovering phenolics from chlorogenate-rich agri-food solid wastes, including potato peels (PPs), eggplant peels (EPPs), and spent filter coffee (SFC). The efficacy of this solvent was compared with other eco-friendly solvents, including aqueous glycerol, aqueous ethanol, and water. The LTTM was demonstrated to be by far the most efficient in extracting chlorogenates and superior or equally efficient with the other solvents in recovering flavonoids. LTTM extracts produced from waste were also more potent radical scavengers, but results on the reducing power were inconclusive. Liquid chromatography-diode array-mass spectrometry analysis showed that the polyphenolic profiles of all waste extracts obtained with the LTTM were rich in caffeoylquinic and p-coumaroylquinic acid conjugates.

Keywords: agri-food waste; antioxidants; eggplant peels; low-transition temperature mixtures; phenolics; potato peels; spent filter coffee

1. Introduction

The production of food generates inevitably large volumes of waste streams from all stages of the food life cycle, beginning from agriculture, up to industrial manufacturing and processing. Concepts of sustainable industrial production, such as the circular economy, are prominent principles for the development of eco-friendly processes, aiming for a "zero waste economy" in which waste is used as raw material for new products and applications. The large amount of waste produced by the food industry represents a great loss of valuable resources and raises serious environmental and economic concerns. However, these residual materials have a great valorization potential through biorefining [1]. Certain agri-food waste is regarded as a rich source of precious substances, which may possess several bioactivities, such as high antioxidant potency [2].

These substances belong mainly to the large polyphenol family, which includes many subclasses with distinct and peculiar pharmacological properties, pertaining to remedies relative to inflammation, cardiovascular disorders, and some types of cancer [3]. Peels from various tissues, *i.e.*, tubers (potato, onion) and fruits (citrus, apple), but also rejected material from plant food processing (e.g., grape

pomace and olive mill wastewater) are cheap and abundant bio-materials and may bear a significant load of bioactive polyphenols, which could be used as natural food additives [4]. This being the case, there has been an enormous number of examinations dealing with the effective valorization of food waste biomass through methods of polyphenol recovery. Techniques involving solid-liquid extraction are the tool of preference in retrieving phenolics from such bioorganic matrices; yet industry faces important challenges for relevant large-scale applications, which must meet specific demands for green production, such as the use of non-toxic and renewable solvents, the cessation of further waste, less energy consumption, and safe, high-quality final products.

In this direction, a new generation of natural and environmentally benign solvents, known as deep eutectic solvents (DES) or low-transition temperature mixtures (LTTMs), is gaining interest. These solvents can be produced using natural non-toxic biomolecules (e.g., organic acids, polyols, salts, *etc.*) by employing mild, simple, and straightforward methodologies, and they may possess unique properties suitable for high-efficiency extractions [5]. Although there has been a notable increase in the use of LTTMs as solvents for natural product recovery [6], the combinations of natural constituents leading to the formation of LTTMs are countless; thus, the search for efficient but also cost-effective LTTMs is imminent.

On such a ground, this study was undertaken to test the efficiency of a newly designed LTTM composed of inexpensive natural materials (glycerol and ammonium acetate) to recover phenolic antioxidants from three types of food waste: potato peels, eggplant peels, and spent filter coffee. These residues were selected because of their peculiar composition that embraces mainly hydroxycinnamate derivatives—the principal compounds of which are chlorogenic acid and the several isomers thereof [7,8]—which display important antioxidant potency and other bioactivities [9]. The extraction efficiency of the LTTM produced here was evaluated and compared with other green solvents, including aqueous glycerol, aqueous ethanol, and water.

2. Materials and Methods

2.1. Chemicals

Solvents used for chromatographic analyses were HPLC grade. Ascorbic acid, 2,4,6-tripyridyl-*s*-triazine (TPTZ) and 2,2-diphenyl-picrylhydrazyl (DPPH•) stable radical were from Sigma Chemical Co., (St. Louis, MO, USA). Glycerol (>99%) and absolute ethanol were from Fisher Scientific (Bridgewater, NJ, USA). Ammonium acetate and aluminium chloride were from Penta (Prague, Czech Republic).

2.2. Low Transition Temperature Mixture (LTTM) Synthesis

For the synthesis of LTTM, a previously reported methodology was used [10]. Briefly, glycerol (hydrogen bond donor (HBD)) was mixed with ammonium acetate (hydrogen bond acceptor (HBA)) at a molar ratio of 3:1 in a glass vial and heated mildly (50 °C) for approximately 15 min until a perfectly transparent liquid was formed. The LTTM was stored in the dark at ambient temperature, where no crystal precipitation was observed over a period of several weeks.

2.3. Agri-Food Wastes

Collection and preparation of spent filter coffee (SFC), eggplant peel (EPP), and potato peel (PP) waste were carried out as described previously [11–13]. All materials were stored in the dark, in sealed plastic containers, at 4 °C.

2.4. Extraction Procedure

A previously described methodology was used [14]. Briefly, the amount of 0.5 g of dry material was mixed in a screw-cap plastic tube with 50 mL of solvent to give a liquid-to-solid ratio ($R_{L/S}$) of 100 mL· g^{-1}, and extracted for 3 h at 80 °C under continuous stirring at 600 rpm. Heating was provided

by an oil bath placed on a thermostated hotplate (YellowLine MST Basic C, Richmond, VA, USA). After the completion of the extraction, the mixture was centrifuged at 10,000 rpm for 20 min, and the clear supernatant was used for all analyses.

2.5. The Determination of Yields in Total Polyphenols (Y_{TP}), Total Chlorogenates (Y_{TCg}), and Total Flavonoids (Y_{TFn})

Yield in total chlorogenates was determined according to a previously published protocol [12]. Results were expressed as mg chlorogenic acid equivalents (CGAE) per g of dry mass. For total flavonoids, a modification of a reported methodology was used [15]. An aliquot of 0.1 mL was mixed with 0.86 mL 35% (v/v) ethanol and 0.04 mL of a reagent containing 5% (w/v) $AlCl_3$ and 0.5 M CH_3COONa. The mixture was left for 30 min at room temperature, and the absorbance was then measured at 415 nm. The concentration of total flavonoids in the extracts (C_{TFn}) was determined as mg rutin equivalents (RtE) per of dry mass using a rutin calibration curve (14.66–293.16 mg$\cdot$ L^{-1}). Yield in total flavonoids was determined as follows:

$$Y_{TFn}\ \left(mg\ RtE\ g^{-1}\ dw\right) = \frac{C_{TFn} \times V}{m}, \quad (1)$$

where V is the volume of the solvent (L), and m is the dry weight of the waste (g). Total polyphenol yield was determined as the sum of Y_{TCg} and Y_{TFn}.

2.6. Antioxidant Activity Assays

Ferric reducing power (P_R) was estimated using the TPTZ methodology [16]. The antiradical activity (A_{AR}) was measured with the DPPH probe, as described elsewhere [17]. P_R and A_{AR} were expressed as µmol ascorbic acid equivalents (AAE) and µmol DPPH per g of dry material, respectively.

2.7. Statistical Analysis

All extractions were repeated at least twice. Determinations were carried out in triplicate, and values were averaged. Correlations and value distributions were determined using linear regression and distribution analysis, respectively, at least at a 95% significance level. Statistics were performed with Microsoft™ Excel 2010 and JMP™ 10.

3. Results and Discussion

3.1. Extraction Efficiency

The LTTM used in this study is heretofore unreported, as ammonium acetate has never been used as a HBA in combination with glycerol. The choice of ammonium acetate was based on its low price and the fact that it is used as food additive (approved by the E.U. as E264), with no reports on any adverse effects on human health. On such a ground, this substance was mixed with glycerol at a molar ratio of 3:1, which has been shown to yield stable LTTMs with sodium acetate serving as the HBA [10]. Furthermore, mixing of glycerol with other HBAs, such as choline chloride, at a ratio of 3:1 resulted in LTTM with reduced viscosity compared with that of glycerol [18], which might also be critical to the extraction process, by increasing the diffusivity of the solute(s) [12]. For this reason, the LTTM was used as an 80% (w/v) aqueous solution to regulate downward its viscosity. Along with this solvent, three other green solvents were tested, including water, aqueous glycerol, and aqueous ethanol, whereas aqueous methanol was used as a control solvent.

The determination of the Y_{TCg} of the extracts generated showed that, for all three wastes used, the LTTM was significantly more efficient ($p < 0.05$), as opposed to water, which displayed significantly lower yields (Table 1). The same conclusion was reached when Y_{TP} was considered, estimated as Y_{TCg} + Y_{TFn} (data not shown). On the other hand, Y_{TFn} did not exhibit the same pattern, since the LTTM was significantly more efficient ($p < 0.05$) only for EPP extraction (Table 2). For PPs, 80% (w/v)

aqueous glycerol was the most potent solvent system in recovering flavonoids, while for SFC all solvents were virtually of equal efficiency, with the exception of 80% (w/v) aqueous glycerol, which gave a low yield ($p < 0.05$).

Table 1. Total chlorogenates (Y_{TCg}) (mg· CGAE· g^{-1}· dw) of the extracts obtained with the solvents tested. Extractions were carried out at liquid-to-solid ratio ($R_{L/S}$) = 100 mL· g^{-1} and 80 °C under stirring at 600 rpm for 3 h.

Solvent	Waste		
	PPs	EPPs	SFC
80% (w/v) aq glycerol	5.49 ± 0.29	21.09 ± 0.73	5.89 ± 0.19
50% (v/v) aq methanol	5.74 ± 0.22	19.67 ± 0.28	9.24 ± 0.20
50% (v/v) aq ethanol	5.05 ± 0.14	19.33 ± 0.31	8.79 ± 0.08
Water	3.53 ± 0.14 [a]	14.30 ± 0.28 [a]	5.29 ± 0.15
LTTM	7.38 ± 0.55 [a]	27.63 ± 0.31 [a]	11.74 ± 0.22 [a]

[a] Denotes statistically different value ($p < 0.05$).

Table 2. Total flavonoids (Y_{TFn}) (mg· RtE· g^{-1} dw) of the extracts obtained with the solvents tested. Extractions were carried out at $R_{L/S}$ = 100 mL· g^{-1} and 80 °C, under stirring at 600 rpm, for 3 h.

Solvent	Waste		
	PPs	EPPs	SFC
80% (w/v) aq glycerol	6.02 ± 0.09 [a]	12.85 ± 0.27	8.70 ± 0.13 [a]
50% (v/v) aq methanol	3.14 ± 0.09	10.13 ± 0.14	12.78 ± 0.25
50% (v/v) aq ethanol	2.68 ± 0.13	9.60 ± 0.14	12.94 ± 0.05
Water	1.98 ± 0.20	9.94 ± 0.60	14.82 ± 0.98
LTTM	4.66 ± 0.05	24.68 ± 0.80 [a]	12.48 ± 0.05

[a] Denotes statistically different value ($p < 0.05$).

Considering these data, it is evident that the LTTM used is an efficient solvent, surpassing the capacity of common conventional solvents such as methanol and ethanol. This argument further concurs with the outcome of a recent examination that demonstrated that the LTTM composed of lactic acid:ammonium acetate (3:1) was particularly effective in the extraction of polyphenols from various medicinal herbs [19]. However, a similar LTTM composed of glycerol:sodium aetate (3:1) did not display higher efficacy compared with aqueous ethanol in the extraction of antioxidant polyphenols from a considerable amount of solid food waste [10].

For PPs and EPPs, it has been demonstrated through detailed investigations that aqueous glycerol and aqueous ethanol have almost identical capacity in extracting phenolics [12,13]. Results on the extraction of waste apple peels were in the same line [20]. For SFC, 3.6% (w/v) aqueous glycerol was shown to afford higher polyphenol yield compared with water [11], evidence that even low glycerol amounts in aqueous solvents may favor polyphenol recovery. The increased yields obtained with solvents composed of aqueous glycerol has been ascribed to the polarity of the medium, which apparently matches that of polyphenolic substances, many of which are rather sparingly soluble in water [16]. However, it should be emphasized that the polarity of a solvent, which may be defined as the capacity of a solvent for solvating various species (molecules or ions), cannot be adequately described based on a single property and therefore cannot be expressed quantitatively [21].

Detailed information on LTTM polarity is particularly limited [22]; thus, only claims could be made regarding the data obtained. The examination of a large number of LTTMs indicated that those composed of a polyol, such as glycerol, may have polarities significantly lower than water, which might explain their higher solubilizing capacity towards phenolics [6]. This is presumably the reason that the LTTM tested in this study displayed much greater efficiency than water. Furthermore, it has been supported that polyphenols may act as the HBD, competing with glycerol in the interactions with the HBA. In cases where LTTMs include a halide ion (e.g., Cl^-), interactions would be hindered

owing to the surrounding of the small anion by the hydroxyl groups of glycerol [23]. However, in the case of ammonium acetate, engulfment of either ammonium or acetate ion by glycerol would be rather limited due to their larger size [19]. Thus, both ions could interact, increasing solubilization of the solute (polyphenols) and hence extraction yield.

3.2. Polyphenolic Profile and Antioxidant Activity

The extracts generated with the LTTM, which had the highest Y_{TP} yields, were analyzed using liquid chromatography-diode array-mass spectrometry. Given the richness of the waste used in chlorogenates, chromatograms were monitored at 320 nm to obtain a representative picture of the chlorogenate profile (Figure 1). PP extracts had a rather poor composition, the main constituent tentatively identified as caffeoylspermine (Table 3). This contrasted with previous data that showed chlorogenic acid to be by far the major polyphenolic constituent [13]. EPP and SFC extracts were dominated by a series of caffeoylquinic but coumaroylquinic derivatives as well, in accordance with earlier findings [11,12]. In PP and EPP extracts, kaempferol rutinoside was also detected, while SFC was characterized by the presence of quercetin rutinoside.

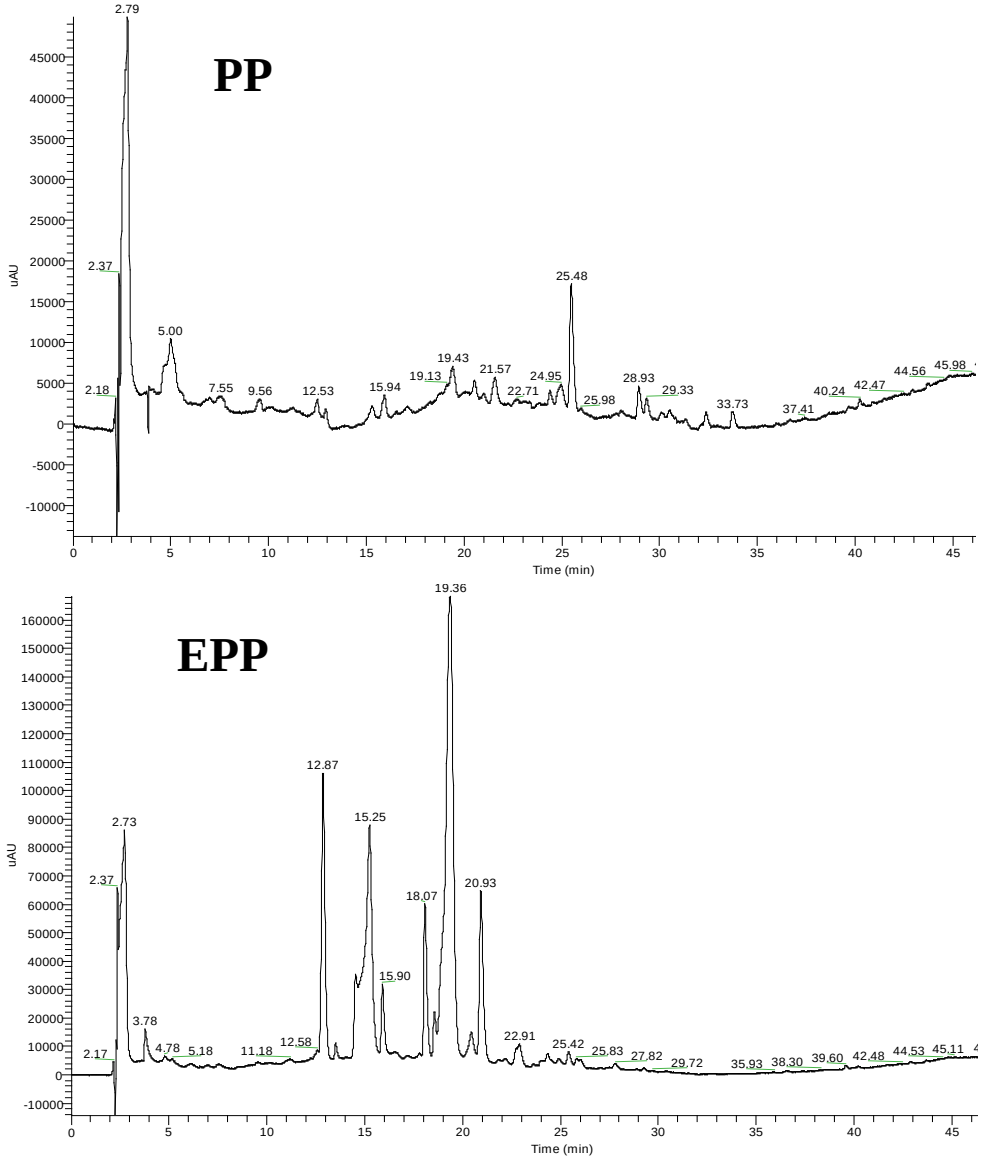


Figure 1. *Cont.*

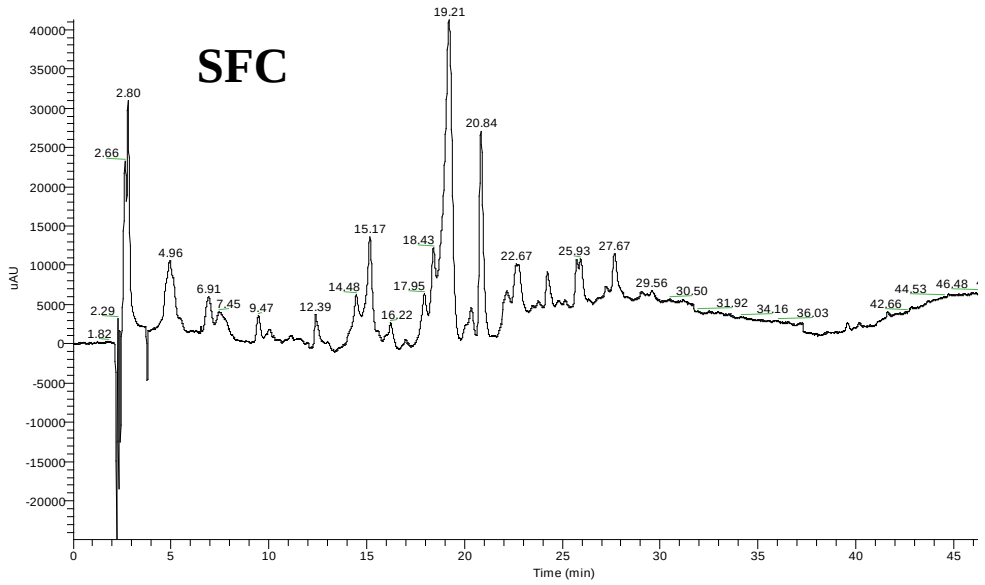


Figure 1. Characteristics chromatograms of the agri-food waste extracts produced with the low transition temperature mixture (LTTM). Monitoring of the eluents was carried out at 320 nm. Assignments: PPs: potato peels; EPPs: eggplant peels; SFC: spent filter coffee.

To assess the antioxidant properties of the extracts produced, two complementary tests were carried out: the antiradical activity (A_{AR}) and the reducing power (P_R). It can be seen in Figure 2 that the PP extracts with aqueous ethanol, aqueous methanol, and LTTM gave almost identical values, and only the water extracts had a lower A_{AR} ($p < 0.05$). The same was recorded for the EPP extracts; however, for SFC, extracts obtained with the LTTM showed a higher A_{AR} ($p < 0.05$). By contrast, the estimation of P_R revealed that, for both PPs and SFC, the extracts obtained with the LTTM were of much lower efficacy ($p < 0.05$), while the corresponding EPPs also had the lowest P_R value (Figure 3). This finding suggested that the extracts with higher Y_{TCg} might display higher radical scavenging potential, but lower reducing effects.

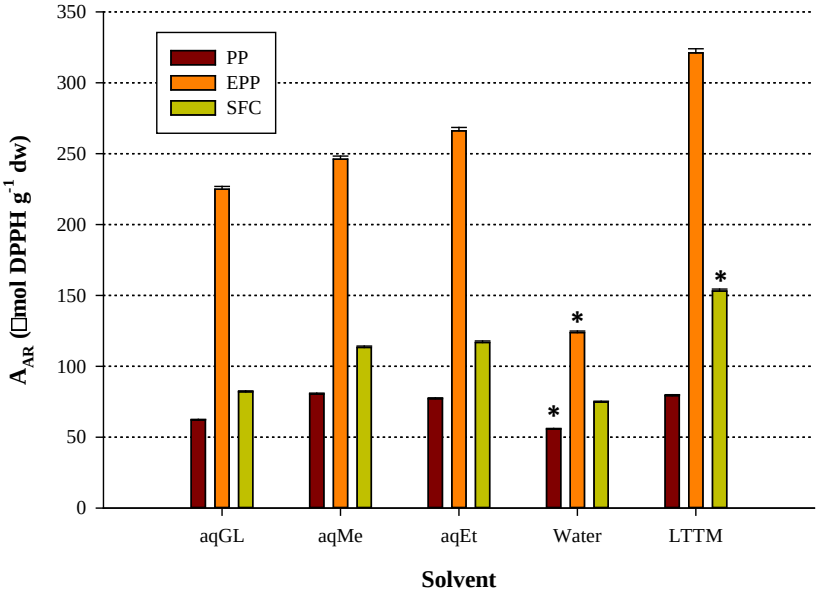


Figure 2. Comparative diagram illustrating the antiradical activity (A_{AR}) of the agri-food waste extracts, produced using the LTTM and other bio-solvents. * denotes statistically different values ($p < 0.05$).

Table 3. UV-Vis and mass spectrometric characteristics of the major polyphenols detected in the LTTM extracts.

No	Rt (min)	UV-Vis	$[M + H]^+$ (*m*/*z*)	Fragment Ions (*m*/*z*)	Tentative Identity	Waste		
						PPs	EPPs	SFC
1	12.87	244, 292, 316	251	-	*p*-Coumaric acid derivative	○	●	○
2	14.48	242, 328	355	163	Caffeoylquinic acid	○	○	●
3	15.17	248, 320	355	163	Caffeoylquinic acid	○	○	●
4	15.25	246, 328	355	377 $[M + Na]^+$, 163	Caffeoylquinic acid	○	●	○
5	15.90	244, 292, 316	339	195	*p*-Coumaroylquinic acid	○	●	○
6	17.95	246, 318	339	195	*p*-Coumaroylquinic acid	○	○	●
7	18.07	246, 292, 318	472	220	N^1-(hydro)caffeoyl-N^8-(hydro) caffeoylspermidine	○	●	○
8	18.43	240, 316	339	195	*p*-Coumaroylquinic acid	○	○	●
9	19.43	246, 328	355	377 $[M + Na]^+$, 163	Caffeoylquinic acid	●	●	●
10	20.84	246, 320	339	195	*p*-Coumaroylquinic acid	○	○	●
11	20.93	246, 328	695	717 $[M + Na]^+$	Caffeic acid derivative	○	●	○
12	21.01	242, 342	595	289	Kaempferol rutinoside	●	●	○
13	22.67	242, 320	339	195	*p*-Coumaroylquinic acid	○	○	●
14	25.48	242, 320	613		Caffeoylspermine	●	○	○
15	25.93	246, 320	517		di-Caffeoylquinic acid	○	○	●
16	27.67	252, 350	611	303	Quercetin rutinoside	○	○	●
17	28.93	238, 322	517		di-Caffeoylquinic acid	●	●	●
18	29.56	242, 320	517	539 $[M + Na]^+$	di-Caffeoylquinic acid	○	○	●

Symbols (●) and (○) signify the presence and absence of a compound, respectively.

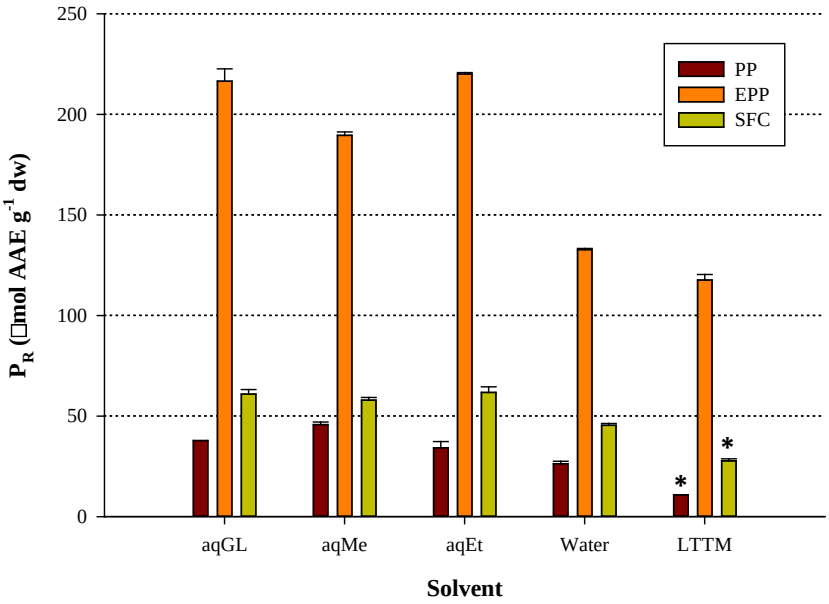
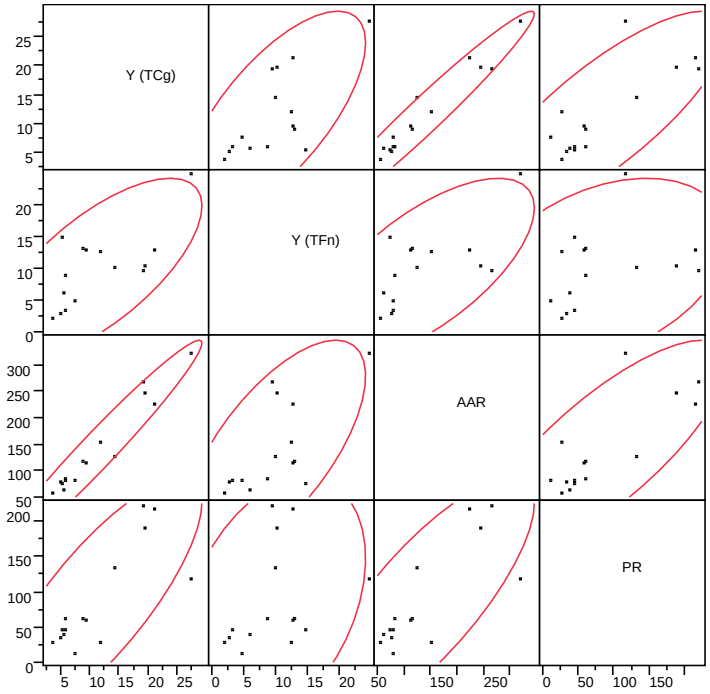


Figure 3. Comparative diagram illustrating the reducing power (P_R) of the agri-food waste extracts, produced using the LTTM and other bio-solvents. * denotes statistically different values ($p < 0.05$).

To test this hypothesis, a multivariate analysis was undertaken, employing pairwise correlations (Figure 4). The analysis indicated that Y_{TCg} was highly correlated with A_{AR} ($p < 0.0001$), but also with P_R ($p = 0.0003$). Correlation of Y_{TFn} was also significant with A_{AR} ($p < 0.0058$), but not with P_R ($p = 0.1991$). This outcome suggested that chlorogenates might indeed be associated mainly with radical scavenging. Such a phenomenon has been observed in plant extracts, where chlorogenic acid was shown to be a major contributor in both the radical scavenging and reducing power [24]. The same conclusion was reached by a study on coffee bean extracts [25] and potato tubers [26]; yet, in a series of extracts from coffee beans of various degrees of roasting, radical scavenging and reducing power values did not coincide [27]. The manifestation of such behavior might lie in certain structural features, such as the catechol moiety. Chlorogenic acid is a rather strong iron chelator [28], and this property could render similar substances a weaker reducing ability. Such a claim was evidenced for caffeic acid-cycteine conjugates [29].

On the other hand, caffeic acid and related derivatives are considered good antioxidants because of the presence of the catechol moiety that gives rise, via hydrogen atom abstraction, to semiquinone radicals, which are well-stabilized by an intramolecular hydrogen bond, hence the relatively small O–H bond dissociation energy [30]. The low O–H bond strength is further reduced by the effect of ring substituents such as the acrylic group (–CH=CH–), which is implicated in the expression of strong antioxidant effects of cinnamic acids and derivatives thereof. Particularly for chlorogenic acid, the major phenolic detected in all three extracts analyzed, it was clearly demonstrated that it can very effectively scavenge various organic radicals, with its efficacy being slightly higher than that of caffeic acid [31]. Subsequent studies confirmed that esters of hydroxycinnamates, such as caffeic, *p*-coumaric, and ferulic acids, were 3–5 times more efficacious than the corresponding free acids, with regard to DPPH scavenging [32].



Pairwise Correlations

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob	
Y (TFn)	Y (TCg)	0.6974	15	0.2881	0.8913	0.0038	*
AAR	Y (TCg)	0.9743	15	0.9223	0.9916	<.0001	*
AAR	Y (TFn)	0.6743	15	0.2475	0.8819	0.0058	*
PR	Y (TCg)	0.8029	15	0.4936	0.9319	0.0003	*
PR	Y (TFn)	0.3513	15	-0.1963	0.7319	0.1991	
PR	AAR	0.7946	15	0.4761	0.9288	0.0004	*

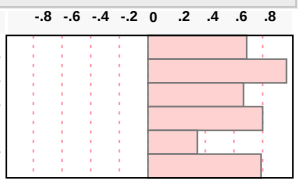


Figure 4. Correlations established following multivariate analysis, between yields (Y_{TCg} and Y_{TFn}) and antioxidant properties (A_{AR} and P_R). The inset table displays statistical values determined for pairwise correlations. Correlations were established at least at a 95% significance level. * denotes statistically different values ($p < 0.05$).

4. Conclusions

A newly synthesized LTTM composed of glycerol and ammonium acetate (molar ratio of 3:1) was tested for its efficiency to extract antioxidant phenolics from chlorogenate-rich agri-food waste, including PPs, EPPs, and SFC. The comparative evaluation with other green solvents, such as aqueous glycerol and aqueous ethanol, showed that the LTTM is more potent to recover chlorogenates, although results on flavonoid yield were inconclusive. The tentative identification of the phenolic substances in the extracts produced with the LTTM indicated that the predominant species are caffeoylquinic and *p*-coumaroylquinic acid conjugates. The outcome from pairwise correlations suggested that the antioxidant effects exerted by the extracts are mainly linked with the amount of the above phenolics.

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Author Contributions: Areti Manousaki and Magdalena Jancheva carried out the experimental work; Spyros Grigorakis performed the LC-DAD-MS analyses; Dimitris P. Makris set up the experimental design, handled and processed raw data and wrote the paper.

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

Nomenclature

A_{AR}	antiradical activity (μmol· DPPH· g^{-1})
C_{TFn}	total flavonoid concentration (mg· RtE· L^{-1})
P_R	reducing power (μmol· AAE· g^{-1})
$R_{L/S}$	liquid-to-solid ratio (mL· g^{-1})
Y_{TCg}	yield in total chlorogenates (mg· CGAE· g^{-1})
Y_{TFn}	yield in total flavonoids (mg· RtE· g^{-1})
Y_{TP}	yield in total polyphenols (mg· g^{-1})

Abbreviations

The following abbreviations are used in this manuscript:

AAE	ascorbic acid equivalents
DPPH•	2,2-diphenyl-picrylhydrazyl radical
dw	dry weight
EPP	eggplant peels
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
LTTM	low-transition temperature mixture
PP	potato peels
SFC	spent filter coffee
TPTZ	2,4,6-tripyridyl-s-triazine

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