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Low-Transition Temperature Mixtures (LTTMs) Made of Bioorganic Molecules: Enhanced Extraction of Antioxidant Phenolics from Industrial Cereal Solid Wastes

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Abstract: Several low-transition temperature mixtures (LTTMs), based on L-lactic acid and amino acids but also choline chloride, were synthesized and screened for their effectiveness in extracting antioxidant phenolics from industrial cereal solid wastes. In most cases, highly efficient LTTMs were those composed of L-lactic acid and choline chloride, but LTTMs composed of L-lactic acid and glycine or alanine also exhibited comparable extraction capacity. The extract from barley bran was shown to express powerful antioxidant activity, which was significantly higher than all the other extracts examined. This fact was attributed to the particularly high content in total flavanols. The data suggested that the most effective solvents, as revealed herein, merit further investigation as very promising means of extracting valuable chemicals from industrial agri-food residues. Additionally, barley bran should be more thoroughly examined for its prospect as a waste source of effective antioxidants, which could be used as nutritional supplements and active cosmetic ingredients.

Keywords: antioxidants; extraction; low-transition temperature mixtures; phenolics; cereal solid wastes

1. Introduction

Renewable materials include a wide range of organic matter that is constantly and abundantly available. In this regard, the biorefinery concept pertains to the production of bioenergy and chemical feedstock from renewable biomass sources and aims at replacing fossil resources. The agri-food sector produces large amounts of solid wastes, which need to be properly managed and handled, to avoid provoking environmental aggravation that would entail serious health and socio-economic consequences. In the case of cereal processing, most grains used for food production are milled to separate the bran and this inevitably results in a significant amount of residual biomass. This waste material, however, contains an array of nutritionally important constituents, including phenolics, vitamins, fiber and minerals, and consumption of bran-based commodities has been claimed to offer several advantages to human health [1,2].

This being the case, this material requires attention as a bioresource of high value–added nutrients and in this respect its potential needs to be thoroughly studied. Financially viable directions for cereal-based biorefineries would therefore embrace a reorientation in the current cereal processing methodologies by implementing parallel production lines for the efficient exploitation of cereal processing wastes [3]. One of the most versatile classes of substances that occur in abundant industrial cereal solid wastes (ICSWs), such as brans, is polyphenols which are secondary plant metabolites with potential health benefits, attributed to their antioxidant activity, but also their antimicrobial, antiviral, and anti-inflammatory effects [4,5]. Hence, ICSWs may be considered as a low-cost resource for the

recovery of phytochemicals, which may have a significant prospect as pharmaceuticals, cosmetic constituents, food additives and nutritional supplements.

Solid-liquid extraction (SLE) is the most common technique for the removal of polyphenols from plant matrices [6], involving phenomena of mass transport, largely affected by factors such as concentration gradients, diffusion coefficients, particle size, temperature and solvent type. Solvent type is one of the major parameters pertaining to the extraction efficiency and, to date, common industrial solvents are of petrochemical origin [7]. The search for sustainable solvents is one of the most significant topics of green chemistry and, in this context, synthesis of new-generation green solvents as replacements for non-eco-friendly ones is of the utmost importance. As such, two principal strategies for green solvent development have been proposed: the replacement of solvents derived from fossil raw materials with others obtained from renewable resources, and the replacement of hazardous solvents with ones that pose no safety or environmental concerns [8].

Low-transition temperature mixtures (LTTMs), also known as deep eutectic solvents (DES), are newly designed liquids, composed of inexpensive, recyclable and non-toxic materials, which can be natural substances (e.g., sugars, organic acids and salts, etc.) [9,10]. LTTM synthesis is eco-friendly, facile and straightforward and properties such as low vapor pressure, absence of flammability and water miscibility make LTTMs ideal solvents for a range of sustainable and environmentally benign applications. Recently, the use of such solvents for the extraction of natural products has been attracting interest, because of their unique potency that allows for extraction yields higher than those achieved with conventional solvents [11]. Furthermore, the numerous substances that can be used for LTTM synthesis dictate a thorough study of their properties to identify the conditions best fitted for a given extraction process.

LTTMs are composed of a constituent termed the hydrogen bond donor (HBD) and another one termed the hydrogen bond acceptor (HBA). Recent investigations with LTTMs composed of glycerol or L-lactic acid (HBD) and sodium acetate (HBA), two low-cost biomolecules, have shown that polyphenol extraction yields were much higher than those obtained with water and were comparable to those obtained with aqueous ethanol [12,13]. On such grounds, the study was carried out to screen combinations of L-lactic acid used as HBD, with the natural amino acids glycine and L-alanine, but also choline chloride, as HBAs (Figure 1) in an effort to identify (i) pairs that provide stable LTTMs and (ii) LTTMs that can afford high extraction yields of antioxidant phenolics from ICSWs.

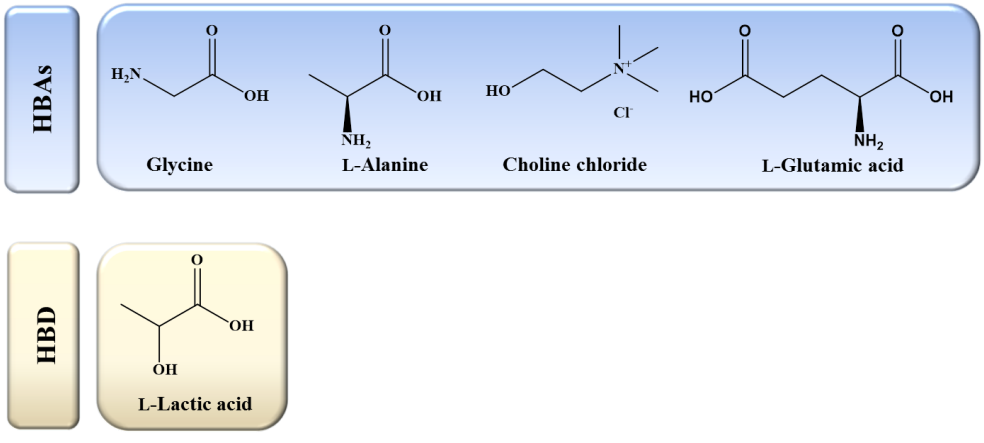

Figure 1. Chemical structures of L-lactic acid and the HBAs used in this study.

2. Materials and Methods

2.1. Chemicals

Choline chloride (>98%) was from Alfa Aesar (Karlsruhe, Germany). L-Lactic acid was from Panreac (Barcelona, Spain). Ascorbic acid, glycine, L-alanine, L-glutamic acid, 2,4,6-tripyridyl-*s*-triazine

(TPTZ), Folin-Ciocalteu reagent, caffeic acid, *p*-dimethylaminocinnamaldehyde (DMACA), catechin, rutin (quercetin 3-*O*-rutinoside) and 2,2-diphenyl-picrylhydrazyl (DPPH$^{\bullet}$) stable radical were from Sigma Chemical Co. (St. Louis, MO, USA). Ferric chloride hexahydrate, absolute ethanol and anhydrous aluminium chloride were from Acros Organics (Bridgewater, NJ, USA). Sodium acetate was from Penta (Prague, Czech Republic).

2.2. LTTM Synthesis

For the synthesis of the LTTMs, previously reported methodologies were used [14]. Briefly, L-lactic acid (HBD) was mixed with either HBA (glycine, L-alanine, L-glutamic acid, choline chloride) at various molar ratios in a stoppered glass vial and heated mildly under stirring, until a perfectly transparent liquid was formed. The LTTMs were stored in the dark at ambient temperature. Details regarding LTTM synthesis are given in Table 1.

Table 1. Combinations of L-lactic acid (LA) with the various HBAs, attempted to generate LTTMs.

Code	HBA	Ratio HBD:HBA	Conditions (*T*, *t*)	Remarks
LA-GA1	L-Glutamic acid	1:1	80–90 °C, 60 min	No interaction
LA-GA3		3:1	80–90 °C, 60 min	No interaction
LA-GA5		5:1	80–90 °C, 60 min	No interaction
LA-GA7		7:1	80–90 °C, 60 min	No interaction
LA-GA9		9:1	80–90 °C, 60 min	No interaction
LA-AL1	L-Alanine	1:1	80–90 °C, 60 min	No interaction
LA-AL3		3:1	80–90 °C, 60 min	No interaction
LA-AL5		5:1	80–90 °C, 60 min	Highly viscous brown liquid
LA-AL7		7:1	80–90 °C, 40 min	Colorless liquid
LA-AL9		9:1	80–90 °C, 40 min	Colorless liquid
LA-AL11		11:1	80–90 °C, 40 min	Colorless liquid
LA-GL5	Glycine	5:1	80–90 °C, 40 min	Colorless liquid
LA-GL7		7:1	80–90 °C, 40 min	Colorless liquid
LA-GL9		9:1	80–90 °C, 40 min	Colorless liquid
LA-CC1	Choline chloride	1:1	80–90 °C, 30 min	Colorless liquid
LA-CC3		3:1	80–90 °C, 30 min	Colorless liquid
LA-CC5		5:1	80–90 °C, 30 min	Colorless liquid

2.3. Industrial Cereal Solid Wastes (ICSW)

The ICSWs used were brans produced from cereal-processing industries, which utilize certified raw materials. Details regarding the ICSWs studied are given in Table 2. The material was received in air-tight packaging and visual inspection affirmed no apparent infections or foreign matter. Brans were ground in a laboratory mill (Tristar, Tilburg, The Netherlands) into a fine powder (average particle diameter $\leq$0.5 mm), placed in screw-cap plastic containers and stored in a dry and dark place for no longer than five days.

Table 2. Codes and origin of the ICSWs used in this study.

Code	ICSW	Certified Raw Material	Origin
WS	Soft wheat bran	*Triticum aestivum*	Pafilis Mills, Corinth, Greece
WH	Hard wheat bran	*Triticum durum*	Pafilis Mills, Corinth, Greece
OT	Oat bran	*Avena sativa*	Strobl Naturmühle Mills, Linz-Ebelsberg, Austria
RY	Rye bran	*Secale cereale*	Strobl Naturmühle Mills, Linz-Ebelsberg, Austria
BL	Barley bran	*Hordeum vulgare* L.	Strobl Naturmühle Mills, Linz-Ebelsberg, Austria
CN	Corn bran	*Zea mays*	Karanikas Mills, Alexandria, Greece

2.4. Extraction Procedure and Sample Preparation

All LTTMs were tested as aqueous mixtures (70%, v/v), and distilled water and 60% (v/v) aqueous ethanol served as control solvents. An amount of 0.4 g pulverized material was mixed with 8 mL of solvent (liquid-to-solid ratio = 20 mL·g^{-1}) in a 15 mL tube and the mixture was vigorously shaken for 5 s to form slurry. Tubes were then placed in a sonication bath (Elma P70, Singen, Germany) and extractions were performed at 55 °C, for 90 min, using the sonication settings described elsewhere [15]. After the completion of the extraction, an aliquot of 1.5 mL of each extract was transferred into 2-mL Eppendorf tubes and centrifuged in a table centrifugator (Hermle, Wehingen, Germany), at 10,621× g, for 10 min. Samples were kept at −20 °C, until analyzed.

2.5. Determinations

Total polyphenol yield (Y_{TP}) was determined with the Folin-Ciocalteu methodology [16] and results were expressed as mg ceffeic acid equivalents (CAE) per g of dry weight. Total flavonoid yield (Y_{TFn}) was assayed with $AlCl_3$ reagent, as previously reported [12] and given as mg rutin equivalents (RtE) per g of dry weight. Yield in total flavanols (Y_{TF}) was measured as μg catechin equivalents (CtE) per g of dry weight, following derivatization with DMACA reagent [17]. The antioxidant activity was estimated by determining the antiradical activity (A_{AR}) and the ferric-reducing power (P_R) of the extracts. Both tests were carried out using published protocols [18].

2.6. Statistics

Extractions with all LTTMs tested were performed at least in duplicate. All the 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 was performed with Microsoft™ Excel 2010 (Microsoft Greece, Anthens, Greece) and and JMP™ 10 (SAS, Cary, NC, USA).

3. Results and Discussion

3.1. LTTM Synthesis

L-Lactic acid (LA) is a natural organic acid that occurs in many foods and may be industrially produced through biotechnological processes [19]. Previous investigations indicated that combining LA as an HBD with the natural amino acid glycine (GL) as an HBA can afford an LTTM with exceptional potency in extracting polyphenolic antioxidants from various medicinal plants [14]. In this line, this study attempted the synthesis of several combinations of LA with GL, but also two other natural amino acids, L-alanine (AL) and L-glutamic acid (GA), to further identify HBD:HBA pairs that could serve as highly efficient solvents. Choline chloride (CC) was also used as a reference material, since it is used as a common HBA [11].

Table 1 fives in detail all the combinations carried out and the conditions employed to generate LTTMs. The LA interaction with CC was facile, yielding perfectly transparent liquids within a relatively short time, which remained stable (no crystallization) for several weeks at room temperature. With reference to GL, in a previous examination it was demonstrated that heating LA with GL at a ratio of 3:1 resulted in the formation of a plastic solid. However, incorporation of water at a ratio of HBD:HBA:water 3:1:3 did yield a stable LTTM. To avoid the addition of water, a combination of LA with GL was performed starting from a ratio of 5:1, which indeed yielded a stable LTTM. Likewise, LA interacted with AL from a ratio 5:1 onward. Regarding GA, any attempt to reach a HBD:HBA ratio of 9:1 did not succeed, pointing out that this particular amino acid does not interact easily with LA.

Based on the above outcome, the LTTMs selected for further testing were LA-AL7, LA-AL9, LA-AL11, LA-GL5, LA-GL7, LA-GL9, LA-CC1, LA-CC3 and LA-CC5. All the LTTMs were used as 70% (*v*/*v*) aqueous solutions because water regulates (i) the polarity of the LTTM, providing, in general, higher extraction yields [11], and (ii) the viscosity of the LTTM, allowing for higher diffusivity. Since solid-liquid extraction is governed by diffusion, increased diffusivity results in a shorter time during which the maximum extraction yield can be achieved [20,21].

3.2. Extraction Efficiency of the LTTMs

All the LTTMs selected were used to extract phenolics from various ICSWs, which may possess variable polyphenolic compositions, thus allowing for a better evaluation of the LTTMs with respect to their extraction efficiency. The evaluation was initially based on a screening, by determining the yield in total polyphenols (Y_{TP}). As can be seen in Table 3, the most efficient solvent for soft wheat bran WS was LA-CC3, which gave Y_{TP} = 5.90 ± 0.41 mg·CAE·g^{-1} dw. This value was significantly higher ($p < 0.05$) compared to Y_{TP} attained with all the other LTTMs, with the exception of LA-CC1. To the contrary, extractions with water and 60% (*v*/*v*) aqueous ethanol were much less efficient, a common finding for almost all ICSWs examined. LA-CC3 was the best solvent for corn bran (CN) extraction as well, yielding 7.33 ± 0.73 mg·CAE·g^{-1} dw. For oat and rye brans OT and RY, significantly higher yields were achieved with LA-CC5, but in both cases there was no statistical difference with the results obtained with LA-CC3. On the other hand, LA-CC5 was the least efficient solvent for the extraction of hard wheat bran (WH). For WH, LA-AL9 and LA-GL7 gave virtually the same Y_{TP} value, which had no significant difference with that seen with LA-GL9. Likewise, for barley bran (BL) both LA-GL7 and LA-AL9 gave comparable yields.

Table 3. Y_{TP} (mg·CAE·g^{-1} dw) of the extracts obtained with the LTTMs tested. Ultrasound-assisted extractions were carried out at $R_{L/S}$ = 20 mL·g^{-1}, for 90 min, at 55 °C.

LTTM	ICSW					
	WS	**WH**	**OT**	**RY**	**BL**	**CN**
LA-GL5	4.15 ± 0.04	3.86 ± 0.35	2.45 ± 0.12	1.93 ± 0.19	3.52 ± 0.07	1.76 ± 0.05
LA-GL7	4.45 ± 0.03	4.81 ± 0.43 $^{\alpha}$	2.42 ± 0.14	2.84 ± 0.28	4.97 ± 0.10 $^{\alpha}$	2.24 ± 0.07
LA-GL9	4.17 ± 0.04	4.46 ± 0.40 $^{\alpha}$	2.36 ± 0.12	2.15 ± 0.21	3.80 ± 0.08	1.94 ± 0.06
LA-AL7	4.11 ± 0.08	3.79 ± 0.08	2.55 ± 0.23	2.26 ± 0.07	3.65 ± 0.15	1.77 ± 0.09
LA-AL9	4.41 ± 0.09	4.82 ± 0.10 $^{\alpha}$	2.51 ± 0.23	2.15 ± 0.06	4.40 ± 0.18 $^{\alpha}$	1.83 ± 0.09
LA-AL11	4.40 ± 0.09	3.94 ± 0.08	2.46 ± 0.22	2.27 ± 0.07	3.85 ± 0.15	1.75 ± 0.09
LA-CC1	4.89 ± 0.34 $^{\alpha}$	3.11 ± 0.16	2.87 ± 0.29	2.16 ± 0.19	1.44 ± 0.13 $^{\alpha}$	2.75 ± 0.28
LA-CC3	5.90 ± 0.41 $^{\alpha}$	4.12 ± 0.21	3.76 ± 0.30 $^{\alpha}$	3.82 ± 0.34 $^{\alpha}$	3.23 ± 0.29	7.33 ± 0.73 $^{\alpha}$
LA-CC5	4.29 ± 0.30	2.39 ± 0.12 $^{\alpha}$	3.81 ± 0.31 $^{\alpha}$	4.82 ± 0.43 $^{\alpha}$	3.34 ± 0.30	2.63 ± 0.26
AE	3.05 ± 0.18 $^{\alpha}$	2.82 ± 0.20 $^{\alpha}$	1.28 ± 0.13 $^{\alpha}$	1.84 ± 0.11 $^{\alpha}$	2.95 ± 0.06	1.70 ± 0.12
W	2.55 ± 0.15 $^{\alpha}$	2.47 ± 0.17 $^{\alpha}$	0.37 ± 0.03 $^{\alpha}$	1.54 ± 0.09 $^{\alpha}$	0.84 ± 0.08 $^{\alpha}$	0.86 ± 0.06 $^{\alpha}$

$^{\alpha}$ Denotes statistically different value ($p < 0.05$).

As an additional step for the evaluation of the extraction efficiency of the selected LTTMs, the extract from each ICSW that displayed the highest Y_{TP} was further analyzed to obtain deeper insight into its polyphenolic composition. In Figure 2, where a comparative plot illustrates the yields in total flavonoids (Y_{TFn}), it is shown that the WH extract obtained with LA-AL9 was the richest, having a Y_{TFn} value of 3.63 mg·RtE·g^{-1} dw. By contrast, the CN extract with LA-CC3 had a Y_{TFn} of only 0.09 mg·RtE·g^{-1} dw. This finding is in accordance with previous studies, which reported a total flavonoid content for wheat bran of 3.20 mg quercetin equivalents g^{-1} dw, significantly higher compared with brans originating from oat, rye and barley [22]. Along the same line, the determination of the yield in total flavanols (Y_{TF}) showed that the BL extraction with LA-GL7 afforded 162.50 µg·CtE·g^{-1} dw and the WH extraction with LA-AL9 gave 140.40 µg·CtE·g^{-1} dw, whereas in the CN extract no flavanols were detected (Figure 3).

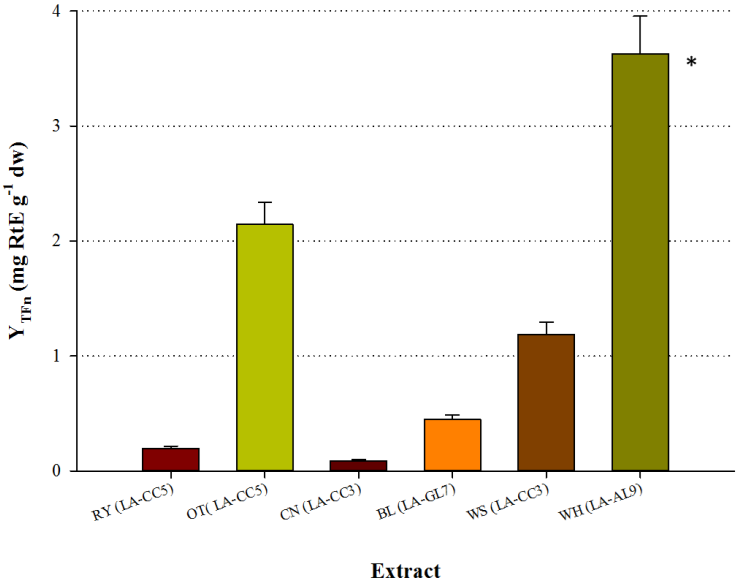

Figure 2. Comparative diagram showing the Y_{TFn} of the richest extracts obtained. Extractions were performed for 90 min, under sonication, at a $R_{L/S}$ = 20 mL·g^{-1}. Asterisk denotes statistically different value ($p < 0.05$).

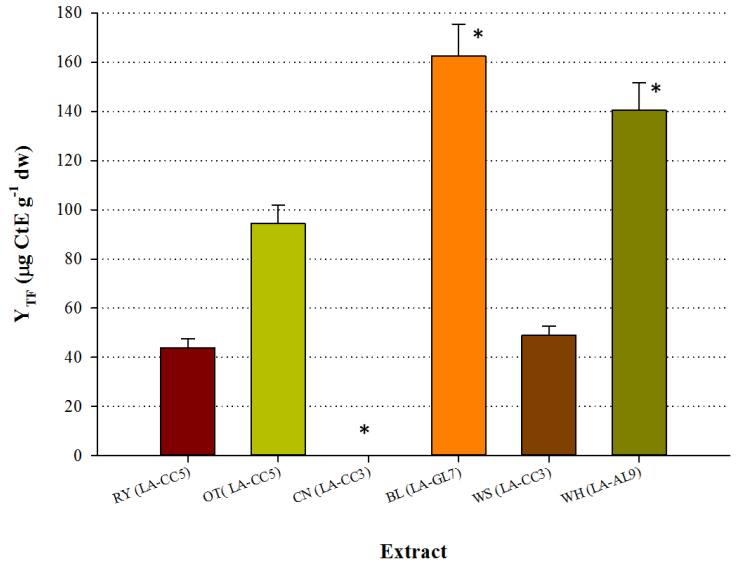

Figure 3. Comparative diagram showing the Y_{TF} of the richest extracts obtained. Extractions were performed for 90 min, under sonication, at a $R_{L/S}$ = 20 mL·g^{-1}. Asterisk denotes statistically different value ($p < 0.05$).

The high extraction efficiency of the LTTMs containing choline chloride as the HBA could be ascribed to interactions of polyphenols with the small anion Cl^-. This is because polyphenols could act as HBDs, competing with lactic acid, as previously proposed [23]. However, in the case of LTTMs containing amino acids, the efficiency observed might be attributed to more hydrogen bonds formed between the polyphenols and the amino groups. It should be stressed, however, that due to a lack of analytical data, such hypotheses remain to be elucidated. On the other hand, increasing the HBD:HBA molar ratio beyond a certain point may result in reduced basicity and limit the interactions between the extractants (polyphenols) and the anion of the HBA, thus affording a lower extraction yield. This is probably the reason for the differences seen with LTTMs composed of the same HBD and HBA, but in different proportions.

3.3. Antioxidant Activity

The differences found regarding Y_{TP}, Y_{TFn} and Y_{TF} are likely to reflect compositional differences among the various brans, which in turn could affect the antioxidant properties of the extracts to an important degree. Indeed, as shown in Figure 4, the BL extract exhibited significantly higher A_{AR} ($p < 0.05$) compared with all the other extracts tested, whereas the WH extract had the lowest performance in this regard. Further, the BL extract also expressed the highest P_R ($p < 0.05$) (Figure 5) This finding is in agreement with previous examinations, which demonstrated that barley bran extracts expressed higher A_{AR} and P_R compared with oat, wheat and rye bran [22].

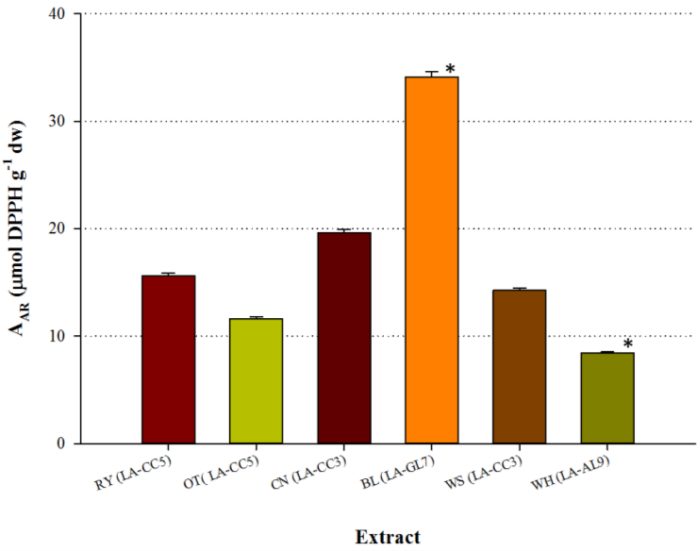

Figure 4. Comparative diagram showing the A_{AR} of the richest extracts obtained. Extractions were performed for 90 min, under sonication, at a $R_{L/S}$ = 20 mL·g^{-1}. Asterisk denotes statistically different value ($p < 0.05$).

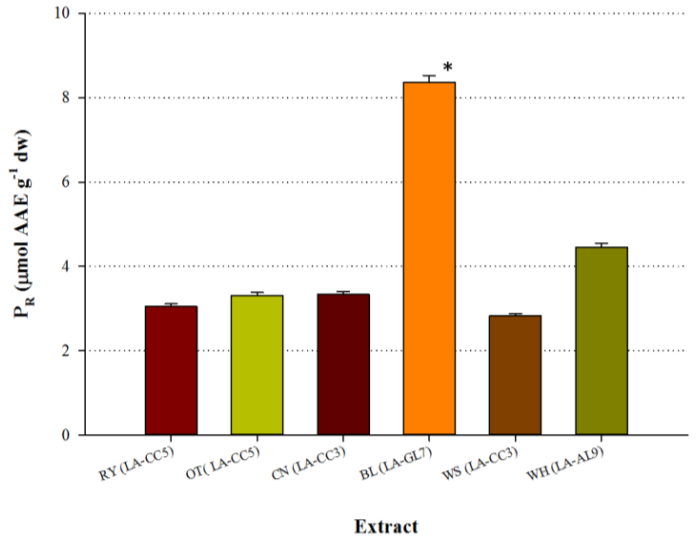

Figure 5. Comparative diagram showing the P_R of the richest extracts obtained. Extractions were performed for 90 min, under sonication, at a $R_{L/S}$ = 20 mL·g^{-1}. Asterisk denotes statistically different value ($p < 0.05$).

Corn bran has been found to contain exceptional amounts of ferulic acid [24,25], which occurs mainly in bound forms [26], but there are no reports for the occurrence of flavanols. This probably

explains the null response of the CN extract to DMACA reagent, which is highly specific to flavanols. Similar results regarding ferulic acid have been reported for oat bran as well [26], but also rye and wheat brans [27]. However, wheat bran contains flavanols, such as catechin [28], and in barley grains the major polyphenol was found to be epigallocatechin gallate (EGCG), accompanied by catechin and epicatechin [29]. On the other hand, the major oat antioxidants have been claimed to be avenanthramides [30,31].

This supporting information is indicative of the variability in the polyphenolic composition of various brans, and the interpretation of the results from the antioxidant tests should rely on such grounds. Thus, BL extracts that had the highest Y_{TF} showed the strongest antioxidant activity, evidence that the most powerful antioxidants occurring in the extracts tested were flavanols. Such a hypothesis would be reasonable, considering that flavanols such as EGCG have been recently classified as extremely effective antioxidants, as opposed to ferulic acid, which was characterized as poorly effective [32]. Therefore, extracts enriched in flavanols could be expected to exert stronger antioxidant activity than those containing higher amounts of ferulic acid. Moreover, the co-occurrence of flavanols and phenolic acids could result in phenomena of antagonism, as demonstrated for ferulic acid and ascorbic acid [33,34] and catechin and ascorbic acid [35]. Since ferulic acid is a weaker antioxidant, its regeneration by flavanols would eventually result in the reduced efficiency of the extracts. This was most likely the reason why WH expressed the lowest A_{AR}, although it had a significantly high Y_{TF}.

4. Conclusions

In this study, several previously unreported LTTMs, synthesized using natural organic substances, were assessed for their effectiveness to extract antioxidant phenolics from industrial solid wastes originating from cereal processing. Following an initial screening, it was shown that combinations of LA with CC at molar ratios 3:1 and 5:1 were highly effective in most cases, but particularly high yields were also obtained for some combinations of LA with GL and AL. Owing to the variability in the polyphenolic composition, as revealed by the estimation of the yield in total flavonoids and total flavanols, the richest extracts were found to display significant differences in the antioxidant activity. The most potent extract was the one prepared from barley bran, using LA-GL7 as a solvent, presumably because of its higher flavanol content. The data suggested that the most effective solvents, as revealed herein, merit further investigation as very promising means of extracting valuable chemicals, such as phenolics and flavonoids, from industrial agri-food residues. Additionally, barley bran should be more thoroughly examined for its prospect as a waste source of powerful antioxidants, which could be used as nutritional supplements and active cosmetic ingredients.

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Author Contributions: Panagiotis Kottaras and Michael Koulianos carried out the experimental work; 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})
P_R	reducing power (μmol·AAE·g^{-1})
$R_{L/S}$	liquid-to-solid ratio (mL·g^{-1})
Y_{TFn}	yield in total flavanols (mg·CtE·g^{-1})
Y_{TFn}	yield in total flavonoids (mg·RtE·g^{-1})
Y_{TP}	yield in total polyphenols (mg·CAE·g^{-1})

Abbreviations

AAE	ascorbic acid equivalents
AL	L-alanine

CAE	caffeic acid equivalents
CC	choline chloride
CtE	catechin equivalents
DMACA	*p*-dimethylaminocinnamaldehyde
DPPH$^{\bullet}$	2,2-diphenyl-picrylhydrazyl radical
dw	dry weight
GA	L-glutamic acid
GL	glycine
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
LA	L-lactic acid
LTTM	low-transition temperature mixture
RtE	rutin (quercetin 3-*O*-rutinoside) equivalents
TPTZ	2,4,6-tripyridyl-*s*-triazine

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