Lignocellulose-Degrading Enzymes: A Biotechnology Platform for Ferulic Acid Production from Agro-Industrial Side Streams

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Abstract: Biorefining by enzymatic hydrolysis (EH) of lignocellulosic waste material due to low costs and affordability has received enormous interest amongst scientists as a potential strategy suitable for the production of bioactive ingredients and chemicals. In the present study, a sustainable and eco-friendly approach to the extraction of bound ferulic acid (FA) has been demonstrated using a single-step EH by a mixture of lignocellulose-degrading enzymes. For comparative purposes of the efficiency of EH, an online SFE-SFC-MS extraction and analysis approach was applied. The experimental results demonstrated up to 369.3 mg 100 g^{-1} FA released from rye bran after 48 h EH with Viscozyme L. The EH of wheat and oat bran with Viscoferm for 48 h resulted in 255.1 and 33.5 mg 100 g^{-1} of FA, respectively. The extraction of FA from bran matrix using the SFE-CO_2-EtOH delivered up to 464.3 mg 100 g^{-1} of FA, though the extractability varied depending on the parameters used. The 10-fold and 30-fold scale-up experiments confirmed the applicability of EH as a bioprocessing method valid for industrial-scale. The highest yield of FA in both scale-up experiments was obtained from rye bran after 48 h of EH with Viscozyme L. In purified extracts, the absence of xylose, arabinose, and glucose as final degradation products of lignocellulose was proven by a HPLC-RID system. Up to 94.0% purity of FA was achieved by SPE using the polymeric reversed-phase Strata X column and 50% EtOH as eluent.

Keywords: biorefining; bran; enzymatic hydrolysis; green-extraction; hydroxycinnamates; sustainability; valorization

1. Introduction

Cereals make a significant contribution to the economy of the EU, and their by-products are thought to be a potential renewable source of high-demand bioactive compounds. According to FAOSTAT (2021) [1], the global cereal production quantity, incl., barley, maize, millet, oats, rice, rye, sorghum, wheat (average) over the last five decades increased by 240.5% from 108.3 million tons (Mt) in 1961 to 368.9 Mt in 2019 [1]. Due to their availability, the by-products are getting enormous attention amongst researchers all over the world as a potential and renewable raw material suitable for the manufacturing of active biomolecules and added-value functional ingredients [2]. However, due to a lack of innovations in grain-derived by-products processing and existed limitations in effective full-fledged transfer of data from scientific institutions to food processing and pharmaceutical industries, grain by-products still are utilized as feedstuff. The presence of cellulose, hemicellulose, and lignin, the way the bioactive compounds are integrated into the matrix, along with adverse effects on technological processes, the application of bran in the food industry plays a secondary role. Valorization of by-products could be done through the application of innovative and green strategies aimed at biomass transformation by extracellularly produced hydrolases such as cellulases, xylanases, and feruloyl esterases, etc.

It has been demonstrated that wheat bran could be used as a raw material for the synthesizes of fumaric acid by enzymatically hydrolyzing (EH) acid pretreated wheat bran with *Rhizopus oryzae* [3]. Bioconversion of wheat bran-derived ferulic acid (FA) through the utilization of a mixture of commercial enzymes and an *E. coli* JM109(pBB1) strain could deliver up to 90.0 mg L^{-1} of vanillin, a flavoring compound that is highly demanded and widely used in the food industry [4]. Within EH of grain-derived by-products, selective production of FA could be achieved by cellulolytic and xylanolytic enzymes that are capable of depolymerizing β-D-(1→4)-glucosidic and β-D-(1→4)-xylosidic bonds of cellulose and hemicellulose polymers, respectively [5]. More recently Ferri et al. [6] proposed a sequential treatment of thermally pretreated wheat bran for the selective release of FA through the application of a blend of proteolytic, amylolytic, and cellulolytic enzymes. The authors highlighted the efficiency of developed technology, since a 40-fold scale-up EH process has given a satisfactory yield of FA. These results were further confirmed by Martín-Diana et al. [7], demonstrating ability of multi-active β-glucanase and β-xylanase enzyme complex Ultraflo XL to release FA from wheat bran under optimal conditions.

Rye bran is less explored, however, likewise to wheat, it can be biorefined and further used as a source of such bioactives as alkylresorcinols, benzoxazinoids, and phenolic acids [8]. This assertion has already been reinforced by Kapreliants and Zhurlova [9], indicating that biomodification of rye bran within stepwise EH of thermally and mechanically pretreated bran material aided in biotechnologically obtaining high-value bioactives, i.e., leucine, arginine, valine. Similarly to wheat, the hydroxycinnamates (HCMs) in the rye bran (FA specifically) are present as components of the outer layer of the kernel, which are located in outer and inner pericarps, seed coats (testa), hyaline, and aleurone layers, and are tightly attached to hemicellulose through ester bonds [10]. The ratio of free to bound FA in rye arabinose-xylose (hemicellulose) fraction is 0.1:1000, indicating a strong binding affinity of this molecule to other macronutrients. The report of Konopka et al. (2014) [11] revealed the presence of 0.0082 mg 100 g^{-1} free FA in whole rye grain flour, pointing to the relative abundance of this phenolic acid in rye grain in relation to other cinnamates. Alkaline-assisted hydrolysis with subsequent solid-liquid or liquid-liquid extraction seems to be a feasible solution to release bound forms. The report of Andreasen et al. [12] indicates the presence of FA in the range of 90.0–117.0 mg 100 g^{-1} in 17 analyzed alkaline-hydrolyzed rye varieties. Inconsistent results, however, were reported by Buksa et al. [13], showing that the content of FA after 24 h alkaline-assisted hydrolysis of a fraction rich in arabinoxylans was found to be considerably lower than that of non-treated samples. The authors pointed out to harsh conditions during hydrolysis had led to the quantitative and qualitative loss of phenolic acids, incl., FA. A similar observation has been made by [14], indicating the formation of 4-vinylguaiacol as the first degradation product of FA that appeared during alkaline hydrolysis.

Although the release of FA using alkaline-assisted hydrolysis of bran material is high, unluckily its production under industrial scale requires substantial input of strong alkalis and acids, however, regarding already existing greenhouse gas emission problems, production and utilization of strong bases or acids would adversely affect human health, the environment, and industry. In a recent study of Juhnevica-Radenkova et al. [14], up to 135.6% of the total alkaline-extractable FA release was achieved from rye bran after EH with a sole multi-enzyme complex Viscozyme L. However, this is the only report highlighting the release of FA during EH with lignocellulose-degrading enzymes from rye bran available so far.

Oats, due to their nutritional composition and availability of multiple bioactive compounds, such as β-glucan, avenanthramides, tocopherols and tocotrienols, phytosterols, phytic acid, and avenacosides, are reported to have beneficial effects on human health, incl. reduction of cardiovascular diseases, type 2 diabetes mellitus, gastrointestinal disorders and cancer risk [15]. It has been stated that among phenolic compounds, FA is found to be the dominant HCM in oat, however, similarly to wheat and rye, is integrated into the matrix of the three-dimensional structure of cellulose, hemicellulose, and lignin, forming strong ester or ether linkages with it [16]. The report of Zhao and Moghadasian [17] reveals that whole oats may contain from 25–35 mg 100 g^{-1} of FA, while oat bran 33.0 mg 100 g^{-1}, however, growing conditions and variety are the main factors determining the amount of FA.

In the context of the "EU Green deal" process, EH eliminates the use of toxic and/ or corrosive chemicals and, due to relative simplicity in operational conditions could represent the future strategy for the production of compounds that are currently manufactured by chemical routs.

The limited information on the recovery of FA and other HCMs from rye, wheat, and oat bran by means of green processing technologies promoted the design of this study, focusing on the evaluation of the release of FA from bran as a result of biorefining accomplished by three lignocellulose-degrading enzymes.

2. Materials and Methods

2.1. Plant Material

Three types of commercial food-grade bran samples were obtained from a local supplier the Ltd. “Voldemars”, separated as rye (*Secale cereále* L.), wheat (*Triticum aestívum* L.), and oat (*Avena sativa* L). Based on morphological evaluation, such hydrological layers as outer pericarp, inner pericarp (cross cells, tube cells), seed coat (testa), hyaline, and aleurone layers with attached starch granules were identified in the bran samples. The proximate composition of the bran samples is shown in Table 1.

Table 1. Nutritional composition of bran by-products derived from rye, wheat and oat grains, g 100 g^{-1} DW.

Major Nutrients Profile, g 100 g^{-1} DW								
						FA, mg 100 g^{-1}		
Type of Bran	**Moisture, %**	**CH**	**Crude Lipids**	**Crude Proteins**	**DF**	**Free**	**Thermally processed**	**Alkali-hydrolyzed**
Rye	11.7± 0.2 ^{a}	30.9 ± 0.5 ^{b}	3.8 ± 0.1 ^{c}	16.9± 0.5 ^{a}	36.0 ± 1.9 ^{b}	19.6 ± 0.6 ^{b}	14.2 ± 0.1 ^{b}	171.2 ± 3.2 ^{b}
Wheat	11.9± 0.2 ^{a}	20.3 ± 0.4 ^{c}	4.5 ± 0.1 ^{b}	16.2 ± 0.4 ^{a}	46.5 ± 2.1 ^{a}	32.1 ± 0.7 ^{a}	19.9 ± 0.1 ^{a}	273.3 ± 4.1 ^{a}
Oat	12.4± 0.3 ^{a}	50.0 ± 0.9 ^{a}	6.7 ± 0.5 ^{a}	14.0 ± 0.7 ^{b}	14.0 ± 1.7 ^{c}	5.7 ± 0.1 ^{c}	10.5 ± 0.1 ^{c}	45.7. ± 1.9 ^{c}

Note: Values are means ± SD values of three replicates (n = e). Means within the same column with different superscript letters (a, b, and c) are significantly different at $p < 0.05$; DW – dry weight; CH – carbohydrates; DF – dietary fiber. Free, thermally processed, and alkali-hydrolyzed refer to the extraction of FA from bran material based on solid-liquid extraction and alkaline-assisted hydrolysis following the methodology described by Juhnevica-Radenkova et al. [14].

2.2. Plant Material Preparation for Alkaline and Enzymatic Hydrolysis and Analysis of Hydroxycinnamates

Each bran sample before EH was ground to reach Ø 0.5 mm particle size using a variable speed rotor mill “KN 295 Knifetec™” (FOSS, Hilleroed, Denmark) and defatted following the methodology described by Radenkovs et al. [18]. Inactivation of native microorganisms and enzymes was done by mixing bran samples with double distilled water (DDW) at a ratio of 1:10 (*w*/*v*) in 25 mL reagent bottles with screw caps (VWR™, International, GmbH, Darmstadt, Germany) with the following subjecting to autoclaving using a digital autoclave with counter-pressure “Raypa, AES 110” (Barcelona, Spain) for 10 min at 121 ± 1 °C temperature and counter pressure 2.0 Pa. After thermal processing, the liquid fraction was decanted for further HPLC analysis (the impact of thermal processing), while solids were freeze-dried using a “Christ Alpha 1-2 LDplus” freeze-drying system (Osterode near Harz, Germany) at −51 ± 1 °C under a vacuum of 0.070–0.080 mBar for 72 h. Dried samples were packed in polypropylene zip-lock silver bags (high-density polyethylene polymer, density 3 mm, Impak Co., Los Angeles, CA, USA) (200 g in each) and stored at a temperature of −18 ± 1 °C until further analysis and use for a maximum of 6 wk. Moisture content was analyzed gravimetrically as proposed by Ruiz [19]

2.3. Chemicals and Reagents

Commercial standards, i.e., caffeic acid (CA), *trans*-isomer of ferulic acid (*t*-FA) and *trans*-isomer of iso-FA (*t*-iso-FA), vanillic acid (VA), vanillin (VN), *p*-coumaric acid (*p*-CA), *trans*-cinnamic acid (*t*-CA), (−)-epicatechin, (±)-catechin, gallic acid (GA), sinapic acid (SA), syringic acid (SIA), protocatechuic acid (PCA), 4-vinylphenol (4-VP), 2-methoxy-4-vinylphenol (4-VG), eugenol (EGN), neochlorogenic (NCGA) and chlorogenic acids (CGA), rhamnose, ribose, xylose, arabinose, sorbose, fructose, mannose, glucose, galactose, sucrose, maltose, lactose, and glycerol were purchased from Sigma-Aldrich Chemie Ltd., (Steinheim, Germany). A standard solution containing mixture of C_4-C_{24} fatty acid methyl esters (FAMEs) with purity ≥99.0% were acquired from Sigma-Aldrich Chemie Ltd. Sodium hydroxide (NaOH), potassium hydroxide (KOH), citric acid ($C_6H_8O_7$), 2,2-diphenyl-1-picrylhydrazyl, sodium citrate dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$), phenolphthalein ($C_{20}H_{14}O_4$), 0.5M trimethylphenylammonium hydroxide solution $(CH_3)_3N(OH)C_6H_5$ (TMPAH) in methanol (MeOH) for GC derivatization were of reagent grade. Ethanol (EtOH), MeOH, acetonitrile (MeCN), formic acid (HCOOH) (puriss r.a.), and ammonium formate (HCO_2NH_4) of LC-MS grade were purchased from Merck KGaA (Darmstadt, Germany). HPLC grade diethyl ether (C_2H_5) and *n*-hexane (C_6H_{14}), pyridine (C_5H_5N) were purchased from Merck KGaA. The ultrapure water was produced using the reverse osmosis PureLab Flex Elga water purification system (Veolia Water Technologies, Paris, France).

2.4. Enzymes

Industrial non-starch polysaccharide (N-SPs) degrading enzyme preparations have been kindly provided by the company Novozymes® (Bagsvaerd, Denmark) for laboratory purposes. Since each preparation selected is a multi-enzyme complex containing various cellulolytic and xylanolytic enzymes, in this study they were applied independently, not as a mixture. A list of enzymes used is given in Table 2.

Table 2. The list of commercial hydrolytic enzymes used in this study.

Commercial Enzyme	Declared Activity	Enzyme Activity	Source	EC number
Viscoferm®	222 FBG g^{-1}	endo-1,4-β-xylanase endo-1,3-(1,4)-β-D-glucanase	*Aspergillus* spp.	3.2.1.8 3.2.1.4
Viscozyme® L	100 FBG g^{-1}	endo-1,4-β-xylanase non-reducing end *α*-L-arabinofuranosidase endo-1,4-β-D-glucanase	*Aspergillus aculeatus*	3.2.1.8 3.2.1.55 3.2.1.4
Celluclast® 1.5 L	700 EGU g^{-1}	endo-1,4-β-D-glucanase	*Trichoderma reesei*	3.2.1.4

Note: EC – enzyme commission; EGU – endoglucanase units; FBG—fungal β-glucanase units.

2.5. Enzymatic Hydrolysis of Rye, Wheat, and Oat Bran

EH of bran samples employing biocatalysts was done in a water bath “SW23” with a capacity of 20.0 L and a horizontal shaking system, and thermostatic and temporal control system (Julabo®, Zalbaha-Hinterglemm, Germany). To estimate the influence of treatment time on the yield of HCMs (FA specifically), the process was done within the range of 12–72 h. The optimal conditions for each enzyme were chosen individually based on the Novozymes® recommendations and following the protocol described by Juhnevica-Radenkova et al. [14]. The EH of N-SPs, i.e., cellulose and hemicellulose was accomplished utilizing three commercially available multi-enzyme complexes, i.e., Viscozyme L, Celluclast 1.5 L, or Viscoferm. For this purpose, 10 mL 0.5 M sodium citrate buffer (pH 4.6) containing 6 FBG mL^{-1} of endo-1,4-β-xylanase (Viscozyme L or Viscoferm), or 10 EGU mL^{-1} of endo-1,4-β-D-glucanase (Celluclas 1.5 L) was added to 1 g of each bran sample. The mixture was then vortexed for 2 min using the “ZX3” vortex mixer (Velp® Scientifica, Usmate Velate, Italy) and incubated in a water bath at 44 ± 1 °C and 100 rpm. The release of HCMs at different time points, i.e., 12, 24, 48, and 72 h of EH was ascertained chromatographically. To terminate the reaction prior to analysis, each aliquot harvested was subjected to ultrasonication at 50 kHz with output wattage of 360 W for 10 min at 25 ± 1 °C using a “Ultrasons” ultrasonic bath (J.P. Selecta®, Barcelona, Spain), followed by centrifugation at 20,160× g for 10 min at 25 ± 1 °C in a “Hermle Z 36 HK” centrifuge (Hermle Labortechnik, GmbH, Wehingen, Germany) and filtration using a 0.22 μm polyvinylidene fluoride (PVDF) hydrophilic membrane filter (Durapore, Millipore, Billerica, MA, USA).

2.6. Hydrolysis of Bran Samples under 10- and 30-fold Scale-Up Process

For FA release from bran matrix, scale-up experiments were performed using the “New Brunswick Bioflo/Celligen 115” bioreactor (Eppendorf, Hamburg, Germany). The process was carried out in a 2.5 L glass vessel at the scale of 10 and 30 g of bran. The same ratio of buffer to substrate was used. During EH, the prepared mixture was continuously stirred at 100 rpm using the Rushton impeller. The pH of the mixture was maintained at 4.6 by the proportional and integral controller which operates peristaltic pumps, assigned to perform acid or base addition. The measurements of pH were done by a gel-filled pH probe (Eppendorf, Hamburg, Germany). The release of HCMs at different time points, i.e., 12, 24, 48, and 72 h, of EH was ascertained chromatographically within 2h. The obtained hydrolysates were ultrasonicated at 50 kHz for 10 min at 25 ± 1 °C and filtered through a 0.22 μm PVDF before chromatographic analysis.

2.7. *Recovery of FA from Bran Hydrolysates by Solid-Phase Extraction*

The FA was extracted from rye bran hydrolysates following a protocol provided by Phenomenex with minor modifications. Briefly, 3 mL of bran hydrolysate was spiked with an internal standard (3,5-dichloro-4-hydroxybenzoic acid), followed by filtration through a 0.45 μm PVDF membrane filter. Recovery of FA was done using solid-phase extraction (SPE) "Strata-X" column (Phenomenex, Torrance, CA, US) filled with styrene-divinylbenzene-based reversed-phase polymer (33 μm, 85 Å, 30 mg 3 mL^{-1}). Conditioning/ equilibration of SPE column was done by 1 column volume of pure MeOH, followed by 1 volume of DDW. The loaded sample was washed with 1 column volume of DDW and a flow-through fraction was collected for further chromatographic work and analysis. Analyte elution was done by 2 volumes of either acidified absolute MeOH or EtOH (2% formic acid *v*/*v*) or their aqueous solutions. Collected eluate fractions were subjected to drying under a gentle stream of N_2 to complete dryness. The dried samples were reconstituted in 1 mL of 80% acidified MeOH (MeOH:DDW:formic acid ratio 80:19:1 *v*/*v*/*v*).

2.8. Plant Material Preparation for SFE-SFC Extraction of Hydroxycinnamates

Duplicate samples of 100 ± 1.0 mg of dried and ground (Ø ≤ 0.5 mm particle size) bran material were weighed into 5 mL stainless steel extraction cells (Tokyo, Japan) with cellulose discs at the bottom and top. The extraction vessel was placed in the rack changer of the SFE apparatus. Liquid CO_2 and EtOH as a co-solvent (modifier) were delivered through pumps into the extraction vessel and changed to supercritical fluid by adjusting temperature and pressure.

Variations in extraction conditions used in experiments to assess the release HCMs are depicted in Table 3, while other parameters are as follows: the extraction solvent consisted of solvent A – supercritical fluid of CO_2 (AGA, Latvia, purity > 99.0); and solvent B (modifier) – EtOH (purity 96.8%); delivered to extraction vessel at flow rate 5.4 mL min^{-1} using a back pressure regulator A – 14.7 MPa and back pressure regulator B – 15.0 MPa both operated at 50 °C.

Table 3. The experimental layout using orthogonal array design.

Experiment No.	Temperature, °C	Pressure, MPa	Co-solvent concentration, %	Extrication time (static to dynamic mode), min
1	**40**	15	10	3:3
2	**45**	15	10	3:3
3	**50**	15	10	3:3
4	**60**	15	10	3:3
5	50	**10**	10	3:3
6	50	**15**	10	3:3
7	50	**20**	10	3:3
8	50	**25**	10	3:3
9	50	15	**7.5**	3:3
10	50	15	**10**	3:3
11	50	15	**15**	3:3
12	50	15	**20**	3:3
13	50	15	10	**1:5**
14	50	15	10	**2:4**
15	50	15	10	**3:3**
16	50	15	10	**4:2**
17	50	15	10	**5:1**

2.9. *The online SFE-SFC Extraction Conditions for Hydroxycinnamates*

Along with alkaline hydrolysis, the protocol described in detail by Juhnevica-Radenkova et al. [14], the online extraction, separation, and analysis of HCMs by means of supercritical-fluid extraction-supercritical fluid chromatography (SFE-SFE) coupled to QqQ-MS 8050 mass selective detector was used in this experiment.

The online SFE-SFC-MS-TQ extraction and analysis was done using the “Nexera UC” SFE-SFC-LC system (Shimadzu Corporation, Tokyo, Japan) with the following configuration: SFE-30A (SFE module), LC-30ADSF (CO_2 deliver pump), LC-40DX3 (modifier deliver pump), DGU-405 (degassing unit), CTO-40AC (column oven), SFC-30A×2 (back pressure adjustment module), Rack Changer II, QqQ-MS-8050 (mass selective detector), SCL-40 (system controller), LabSolutions Insight LC-MS version 3.7 SP3 (workstation), shown in Figure 1.

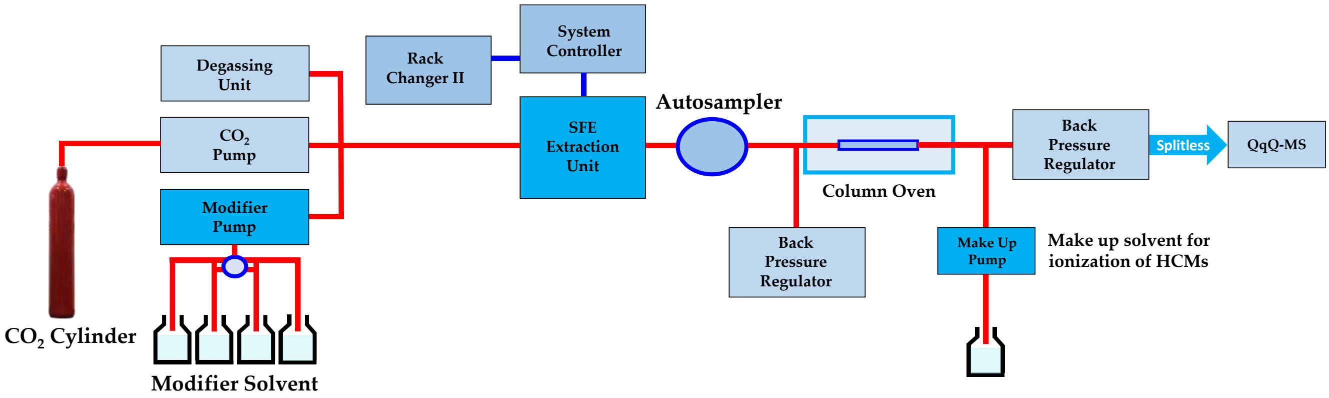

Figure 1. Schematic representation of the online supercritical fluid extraction-supercritical fluid chromatography-mass spectrometry (SFE- SFC-MS-TQ).

2.10. *The Online SFE-SFC-MS-TQ Analytical Conditions for Hydroxycinnamates*

Chromatographic separation of HCMs was carried out using a reversed-phase Shim-pack UC-RP column (5.0 μm, 250 × 4.6 mm; Tokyo, Japan) operating at 45 °C and a flow rate of 1.0 mL min^{-1}. The mobile phase used was supercritical fluid of CO_2 (A) and acidified MeOH (0.002 % formic acid, *v*/*v*) with 5 mM ammonium formate (B). Separation of HCMs was done using the following gradient conditions: elution started with 10% B to obtain 40% B at 11 min, 60% B at 15 min, 10% B at 17 min, with the following 2 min wash and equilibration. A make-up solution consisting of 5 mM ammonium formate in MeOH was delivered with a make-up pump after column separation to promote HCMs ionization. Data was acquired using a LabSolutions Insight software, which was also used for instrument control and processing. The system was operated using both negative and positive ion polarity, and data was acquired in centroid and profile mode, with a data storage threshold of 5000 absorbance for MS. The operating conditions were as follows: detector voltage – 1.8 kV, conversion dynode voltage – 10.0 kV, interface voltage – 4.0 kV, interface temperature – 300 °C, desolvation line temperature – 250 °C, heat block temperature – 400 °C, nebulizing gas – argon (Ar, purity–99.9%,) at flow – 3.0 L min^{-1}, heating gas – carbon dioxide (CO_2, purity – 99.0%,) at flow – 10.0 L min^{-1}, drying gas – nitrogen (N_2, the drying gas was separated from the air using a nitrogen generator system from Peak Scientific Instruments Ltd. (Inchinnan, Scotland, UK, purity – 99.0%) at flow – 10.0 L min^{-1}. All HCMs were monitored in the scheduled multiple reaction monitoring (MRM) mode. A list of all MRM transitions, collision energy, Q1, Q3, and dwell time for phenolic compounds is depicted in Table 4.

2.11. The HPLC-ESI-QqQ-MS Analytical Conditions for Phenolics

The analyses were carried out using a Shimadzu series Nexera UC SFC-SFE-LC system (Tokyo, Japan) coupled to a triple quadrupole (QqQ) mass selective detector MS-8050 (Tokyo, Japan) with an electrospray ionization interface (ESI). Sample of 1 μL was injected onto a reversed-phase Shim-pack UC-RP column (5.0 μm, 250 × 4.6 mm; Tokyo, Japan) operating at 45 °C and a flow rate of 1.0 mL min^{-1}. The mobile phases used were acidified DDW (0.002% formic acid, *v*/*v*) supplemented with 5 mM ammonium formate (A) and acidified MeOH with 5 mM ammonium formate (0.002% formic acid, *v*/*v*) (B). Separation of compounds was done using the following gradient conditions: elution started with 5% B to obtain 10% B at 5 min, 60% B at 12–15 min, 10% B at 18 min. Furthermore, MeOH injections were included every three samples as a blank run to avoid the carry-over effect. Data acquisition, analysis, and processing were done similarly as for the online SFE-SFC-MS-TQ analytical conditions for hydroxycinnamates (section 2.10.).

2.12. The HPLC-RID Analytical Conditions for Carbohydrates

The quantitative analysis of mono- and disaccharides, the hydrolysates along with appropriate calibration standards were run on a Waters Alliance HPLC system (model No. e2695) equipped with a 2414 RI detector and a 2998 column heater (Waters Corporation, Milford, MA). Chromatographic separation was done on an Altima Amino (4.6 × 250 mm; 5 μm; Grace™, Columbia, MD, USA) column. A column and flow cell temperature was maintained at 30 °C. A mixture of DDW and CH_3CN (80:20, *v*/*v*) was used as a mobile phase in isocratic mode. The flow rate of the mobile phase was 1.0 mL min^{-1}. The injection volume was 15 μL. System control, data acquisition, analysis, and processing were done by Empower 2 Chromatography Data Software (Waters Corporation).

Table 4. MRM transitions, collision energy, Q1, Q3 and dwell time for investigated hydroxycinnamates.

Compound	Retention time, min	Molecular formula	Ionization mode	MRM transitions	Q1 Pre Bias, V	Collision energy, V	Q3 Pre Bias, V	Dwell time, msec
Eugenol	1.953	$C_{10}H_{12}O_2$	$[M+H]^+$	165.0000→69.0000	-11.0	-22.0	-15.0	20.0
				165.0000→109.0000	-11.0	-24.0	-13.0	20.0
				165.0000→137.0500	-11.0	-13.0	-16.0	20.0
Gallic acid	9.033	$C_7H_6O_5$	$[M-H]^-$	169.0000→124.9000	12.0	17.0	10.0	20.0
				169.0000→78.9500	12.0	24.0	15.0	20.0
				169.0000→68.9000	12.0	22.0	11.0	20.0
Neochlorogenic acid	10.245	$C_{16}H_{18}O_9$	$[M-H]^-$	353.1000→191.0500	13.0	22.0	20.0	20.0
				353.1000→135.0000	13.0	31.0	12.0	20.0
				353.1000→179.0500	13.0	19.0	10.0	20.0
Protocatechuic acid	11.515	$C_7H_6O_4$	$[M-H]^-$	153.2000→108.9500	10.0	16.0	20.0	20.0
				153.2000→107.9500	10.0	24.0	22.0	20.0
				153.2000→91.0500	10.0	26.0	17.0	20.0
Chlorogenic acid	12.044	$C_{16}H_{18}O_9$	$[M-H]^-$	353.1000→191.1000	19.0	22.0	20.0	20.0
				353.1000→85.0500	13.0	43.0	16.0	20.0
				353.1000→127.0000	13.0	36.0	10.0	20.0
(+)-Catechin	12.703	$C_{15}H_{14}O_6$	$[M-H]^-$	288.9500→245.0000	14.0	15.0	14.0	20.0
				288.9500→109.0000	14.0	26.0	19.0	20.0
				288.9500→123.0000	14.0	31.0	10.0	20.0
(−)-Epicatechin	12.709	$C_{15}H_{14}O_6$	$[M-H]^-$	289.0500→245.0000	14.0	16.0	14.0	20.0
				289.0500→109.0000	14.0	26.0	20.0	20.0
				289.0500→123.0000	14.0	30.0	22.0	20.0
Syringic acid	13.033	$C_9H_{10}O_5$	$[M-H]^-$	197.1500→182.1000	20.0	15.0	10.0	20.0
				197.1500→122.9500	17.0	24.0	19.0	20.0
				197.1500→95.1000	12.0	31.0	18.0	20.0
Vanillic acid	13.248	$C_8H_8O_4$	$[M-H]^-$	167.0500→152.0000	12.0	18.0	30.0	20.0
				167.0500→107.9000	12.0	19.0	20.0	20.0
				167.0500→123.0000	12.0	14.0	19.0	20.0
Vanillin	13.366	$C_8H_8O_3$	$[M+H]^+$	152.9500→65.1000	-10.0	-24.0	-24.0	20.0
				152.9500→93.0500	-10.0	-16.0	-20.0	20.0
				152.9500→125.1000	-10.0	-15.0	-15.0	20.0

Table 4 continuation.

Compound	Retention time, min	Molecular formula	Ionization mode	MRM transitions	Q1 Pre Bias, V	Collision energy, V	Q3 Pre Bias, V	Dwell time, msec
4-Vinylphenol	13.747	C_8H_8O	$[M+H]^+$	121.0500→77.2000	-20.0	-23.0	-16.0	20.0
				121.0500→91.2000	-21.0	-19.0	-18.0	20.0
				121.0500→51.2000	-22.0	-36.0	-21.0	20.0
Caffeic Acid	13.714	$C_9H_8O_4$	$[M-H]^-$	179.1500→135.0000	12.0	18.0	25.0	20.0
				179.1500→134.0000	12.0	25.0	24.0	20.0
Sinapic acid	14.361	$C_{11}H_{12}O_5$	$[M+H]^+$	224.9000→207.1000	-15.0	-10.0	-16.0	20.0
				224.9000→161.0000	-10.0	-10.0	-18.0	20.0
				224.9000→91.1500	-15.0	-27.0	-21.0	20.0
trans-Isoferulic acid	14.728	$C_{10}H_{10}O_4$	$[M+H]^+$	194.9000→177.1000	-13.0	-11.0	-13.0	20.0
				194.9000→131.0000	-13.0	-11.0	-15.0	20.0
				194.9000→89.1500	-13.0	-32.0	-19.0	20.0
trans-Ferulic Acid	14.733	$C_{10}H_{10}O_4$	$[M-H]^-$	193.0500→134.0000	10.0	18.0	23.0	20.0
				193.0500→178.0500	10.0	15.0	15.0	20.0
para-Coumaric acid	14.818	$C_9H_8O_3$	$[M-H]^-$	163.0500→119.0500	11.0	16.0	21.0	20.0
				163.0500→93.0500	12.0	31.0	17.0	20.0
2-Methoxy-4-vinylphenol	17.972	$C_9H_{10}O_2$	$[M+H]^+$	150.8000→91.2500	-10.0	-22.0	-19.0	20.0
				150.8000→65.2000	-25.0	-32.0	-28.0	20.0
trans-Cinnamic acid	18.600	$C_9H_8O_2$	$[M+NH4]^+$	165.0000→101.2000	-12.0	-12.0	-20.0	20.0
				165.0000→69.2000	-12.0	-23.0	-29.0	20.0
				165.0000→133.2500	-11.0	-10.0	-15.0	20.0

The first MRM transitions found were used for qualitative analysis, while the second or third for quantitative.

2.13. Preparation of the Lipid Fraction by Alkaline-Assisted Hydrolysis and Liquid-Liquid Extraction

For the hydrolysis of bran matrix and release of bound forms of fatty acids, the 10% (*w*/*v*) KOH dissolved in 80% MeOH (MeOH:DWW ratio 80:20 *v*/*v*) was used. In excess of MeOH, this method allows the process of hydrolysis and release of fatty acids to be done more efficiently. Triplicate samples of 5 ± 0.1 g of ground bran were weighed in 25 ml reagent bottles with screw caps. For the hydrolysis 20 mL of prepared methanolic KOH was added to bran and the mixture was subjected to incubation in a water bath "TW8" (Julabo®, Saalbach-Hinterglemm, Germany) at 65 °C for 3 h. After hydrolysis the release of fatty acids from the salt form was done by shifting the pH of the medium from alkaline to acidic by adding 6 M HCl until the pH was 2.0. The extraction of the lipophilic fraction was accomplished by liquid-liquid phase separation using *n*-hexane as a solvent. 10 mL of *n*-hexane was added to the prepared hydrolysates (total 15 mL), followed by vortex-mixing for 1 min and separation of the layers by centrifugation at 4500 rpm (3169× g) for 10 min in a "Sigma, 2-16KC" centrifuge (Osterode near Harz, Germany). The top *n*-hexane layer was separated and collected. The extraction procedure was repeated three times. The resulting lipophilic fraction (30 mL) was further evaporated using a "Laborota 4002" rotary evaporator (Heidolph, Swabia, Germany) at 65 °C and the dry fraction was then re-dissolved in 2 mL of pyridine, filtered through a polytetrafluoroethylene hydrophobic membrane filter with a pore size 0.45 μm (VWR™, International, GmbH.) The filtrates were quantitatively transferred to 22 mL glass headspace chromatography bottles for further chromatographic work and analysis.

2.14. Preparation of Fatty Acids for GC/MS Analysis

TMPAH reagent was used as methylation agent of the polyfunctional groups to obtain volatile fatty acid derivatives. The methylation procedure was performed according to the protocol described in the America Society for Testing and Materials [20]. Briefly, 5 μL aliquot of lipid fraction was taken from the separated lipophilic fraction and 5 μL of 1% phenolphthalein indicator ($C_{20}H_{14}O_4$:EtOH ratio 1:99 *w*/*v*), 6 μL of 0.5 M TMPAH reagent and 930 μL of methanol:diethyl ether (MeOH:Et_2O ratio 50:50 *v*/*v*) were added to the vial. The resulting pink mixture was vortex-mixing for 1 min and kept in a GC oven at 60 °C for 30 min. The mixture was cooled to room temperature and further used for FAMEs analysis on GC/MS system. Quantification of compounds was done using a standard solution of C_4-C_{24} FAMEs, building calibration curves for each compound individually.

2.15. The GC Conditions for FAMEs Analysis

The analysis of fatty acid methyl esters (FAMEs) was carried out on a "Clarus 600" system PerkinElmer, Inc. (Waltham, MA, USA) equipped with a quadrupole analyzer "Clarus 600 C" mass-selective detector (Waltham, MA, USA). The chromatographic separation of FAMEs was done using "Trace™ TR-FAME" (Thermo Fisher Scientific, Waltham, MA USA) column with a cyanopropylphenyl-based stationary phase (50 m x 0.22 mm, sorbent thickness – 0.25 μm) specifically designed for the separation of *cis*- and *trans*- isomers of FAMEs (Supplementary Figure S1). The injector temperature has been set to +280 °C; automatic injection using an autosampler, injection volume 0.5 μL; split ration 4:1. The initial oven temperature was maintained at 70 °C for 2 min, then raised to 150 °C (rate of 20 °C min^{-1}), then increased to 250 °C (rate of 4 °C min^{-1}). Helium (ultra-high purity 5.0 grade – 99.999%) was used as a carrier gas at the constant flow rate of 1.0 min with the split ratio n:1. The total separation time was 31.00 min. The analysis was performed in triplicate.

2.16. The MS Conditions for FAMEs Detection

Detector mode: Electron impact ionization was at 70 eV; ion source temperature: +230 °C; inlet temperature was +280; capture time starting from 6.5 min (1.7 scan s^{-1}); ion multiplier: 240 V; and ion *m*/*z* interval: 41–500 atom mass units (AMU) for FAMEs.

2.17. Statistical Analysis

The results obtained are shown as means ± standard deviation of the mean from three replicates (n = 3). The p-value < 0.05 was used to denote significant differences between mean values determined using one-way analysis of variance (ANOVA) and the Duncan's multiple range test done using the assistance of IBM® SPSS® Statistics program 20.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Release of FA from Bran Using Enzyme-Assisted Hydrolysis

In the field of phytochemical research, the occurrence of ferulic acid (FA) and its isomers represents an emerging topic. Much attention has been paid to this issue, chiefly regarding the health benefits of various grain-derived products and by-products containing HCMs [21]. In nature, FA appears as *cis*- and *trans*- forms of two stereoisomers which present mainly in covalently bound forms within cell wall components [22]. According to Palafox-Carlos et al. inference [23], the dietary fiber-bound complexes form groups of compounds called non-extractable polyphenols. The release of these compounds from plant matrix varies depending on the complexity of the structure as well the ways in which compounds and dietary fiber are interlinked [5]. Since nearly 97% of FA in cereal bran is bound with various macromolecules forming cross-links, the efficient release/ extraction of this compound presents a challenge. The alkaline hydrolysis of complex agro-industrial by-products was reported as an effective tool for the extraction of FA and other HCMs. However, concerning environmental pollution matters and governmental intentions outlined in the EC Directive 2010/75/EU [24], aiming to reduce the negative impact of industrial toxic emissions on ecosystems creates an additional demand for developing green technologies for the production of chemicals from renewable sources. More recently, Martín-Diana et al. [7] introduced a novel strategy to produce ingredients rich in FA by means of EH done by multi-enzyme complex Ultraflo XL of thermally and pressure pretreated wheat bran. Following the process developed, 100 g of processed wheat bran could deliver up to 248.94 mg of FA. Based on the HPLC-ESI-QTOF/MS approach the authors managed to detect the presence of both *cis*- and *trans*- FA isomers in their hydrolysates.

To establish the ability of lignocellulose-degrading enzymes to release bound FA forms from bran matrices, incl., rye, wheat, and oat, the EH with a single process step was used in this study. In this set of experiments, bran samples underwent 12 to 72 h EH solely by non-starch polysaccharide degrading enzymes (N-SPs), i.e., Viscozyme L, Celluclast 1.5 L, and Viscoferm. The highest yield of t-FA in all cases of enzymes applied was obtained from rye and wheat bran after 48 h of EH, except for wheat bran and Viscozyme L, where the highest yield was acquired already after 24 h (Figure 2A and 2B).

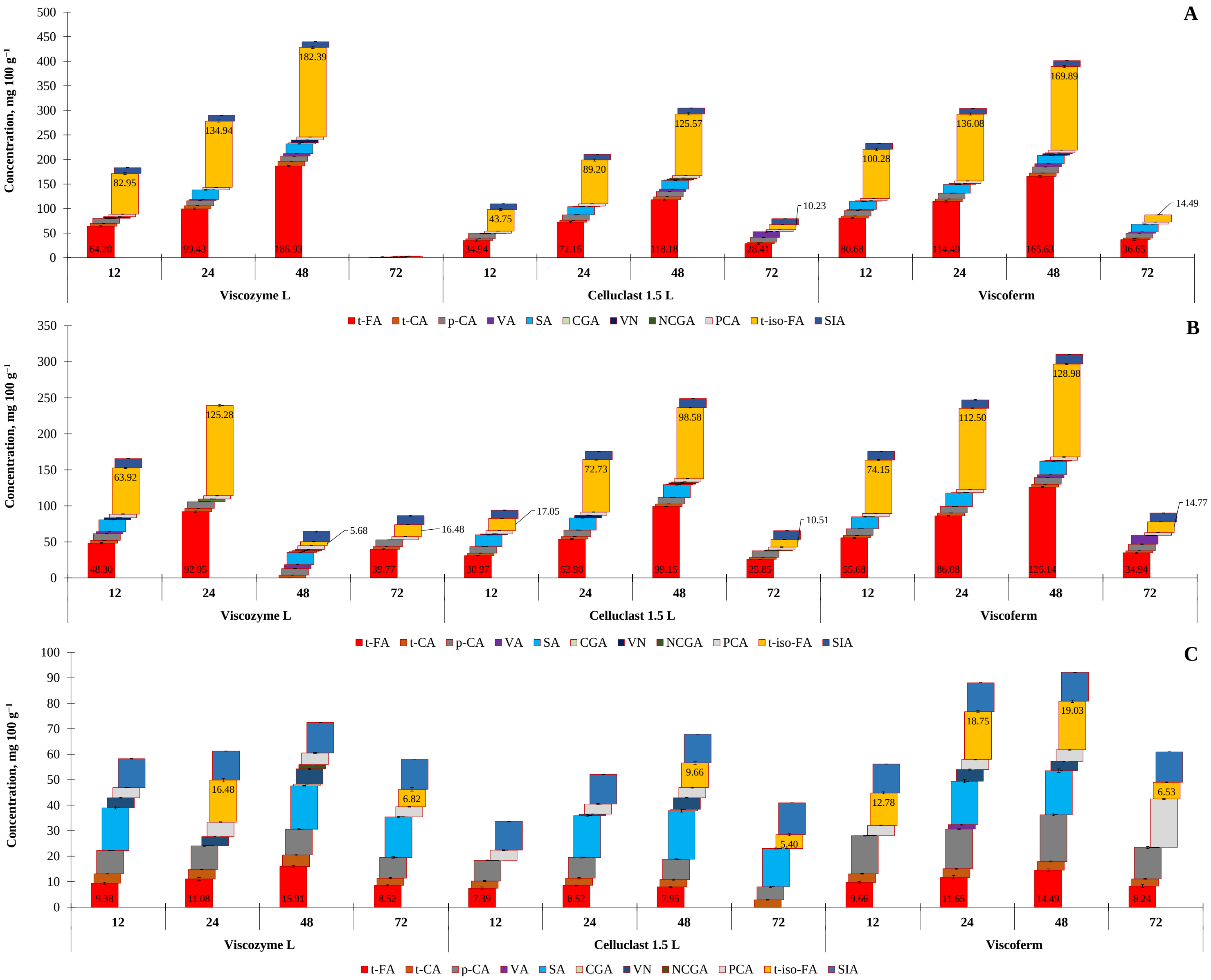

Figure 2. The release of FA and other HCMs using enzymatic hydrolysis of rye (A), wheat (B), and oat (C) bran applying three multi-enzyme complexes, i.e., Viscozyme L, Celluclast 1.5 L, and Viscoferm, mg 100 g^{-1} DW. Note: Values are means ± SD values of triplicates (n = 3).

However, upon EH, a significant loss of *t*-FA by 16.5–37.2% and 18.7–99.4% was observed for rye and wheat bran, respectively, indicating further degradation process caused by thermal decarboxylation of HCMs as revealed by Ohra-Aho et al. [25]. This observation is reinforced by the presence of 2-methoxy-4-vinylphenol (4-VG) (4-vinyl guaiacol) and 4-allyl-2-methoxyphenol (eugenol), the degradation products of FA and Klason lignin, respectively (Figure 3A and 3B).

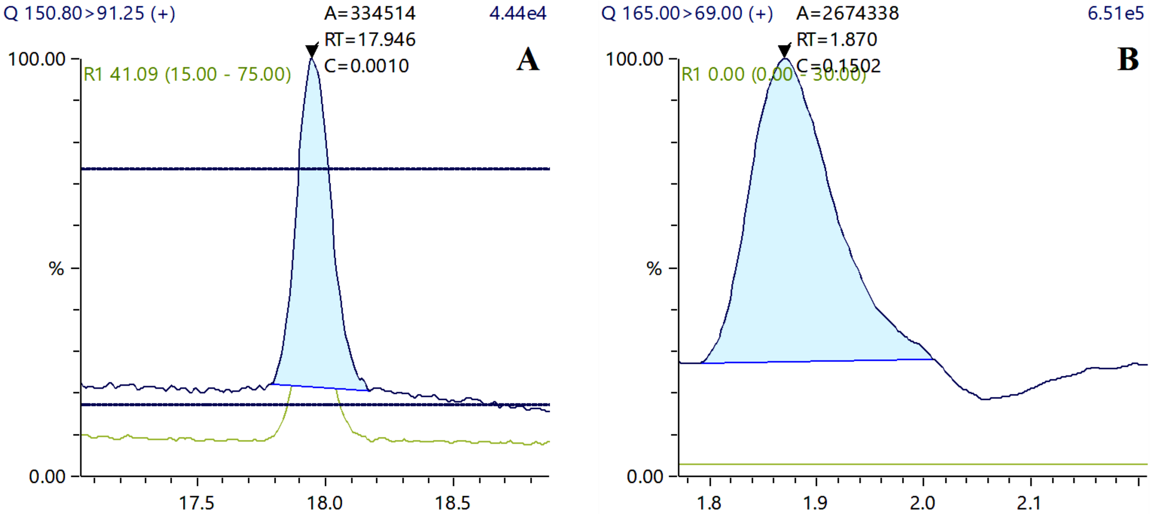

Figure 3. Extracted ion chromatograms (EIC) plotted for the degradation products of 2-methoxy-4-vinylphenol (A) and 4-allyl-2-methoxyphenos (B).

Among the hydrolytic enzymes examined, the advantage of Viscozyme L above other enzymes was noticed during the processing of rye bran. This observation is also established by Mahmoudi et al. [26], working with the enzyme-assisted extraction of bioactives from sweet basil by-products. However, conducting EH of wheat bran with multi-enzyme complex Viscoferm, up to 1.4-fold higher yield of *t*-FA was reached than during EH with Viscozyme L. It is worth noting, though, that the selective MRM-MS-based approach revealed no presence of *t*-FA in wheat bran hydrolysates that underwent EH with Viscozyme L for 48 h. The absence of *t*-FA in hydrolysates can be explained by the ability of *trans*- isomer of FA to be isomerized to *cis*- form under UV irradiation that, perhaps, took place during the next 24 h of EH [27]. Notably, a smaller release of *t*-FA was found in oat bran (Figure 2C), but, along with *t*-FA, an equivalent yield of other HCMs was detected in obtained hydrolysates. The concentration of *t*-FA was almost 11.7-fold and 7.9-fold lower than that released from rye and wheat bran, respectively. A similar amount of *t*-FA released after alkaline-assisted hydrolysis of oat bran has been reported by [28].

The second prevalent HCM isolated from bran hydrolysates and further successfully quantified was *t*-iso-FA. The concentration of *t*-iso-FA in bran hydrolysates fluctuated from 1.26 to 182.4 mg 100 g^{-1} (on a dry weight basis) depending on the enzyme and the duration of EH used. Among the 3 hydrolytic enzymes screened, the highest yield of *t*-iso-FA, similarly to *t*-FA, was obtained in rye bran samples subjected to 48 h EH with multi-enzyme complex Viscozyme L. Considerably smaller release of *t*-iso-FA from rye bran matrix was observed after 48 h EH with Viscoferm, though the amount was 1.3-fold higher than the Celluclast 1.5 L does. The presence of *t*-iso-FA in the grains and grain-derived fractions of such crops as wheat, oats, barley, corn, red rice, and rye has already been confirmed by [29–31], indicating that rye contains the highest amount, but the ratio of free to bound *t*-iso-FA is 10:90. However, the amounts described herein are 4.4- and 28.4-fold higher than that previously reported for wheat and oat bran, respectively.

The most available literature on clinical trials refers to *t*-iso-FA derived from *Cimicifugae rhizoma*, a medical herb with multiple well-documented health-promoting benefits [32,33], although little attention has been given to other sources of *t*-iso-FA. However, despite the beneficial health effects outlined in the report of Li and Yu [34], there are little generalized information about the exact concentration of *t*-iso-

FA. It was hypothesized that the grain-derived hydrolysates discussed herein may have the same beneficial health effects, and could be used as a remedy in the prevention and treatment of various ailments which is also supported by [31].

The SA is the third HCM the presence of which was confirmed in all bran hydrolysates obtained by selected enzymes. The advantage of using Viscoferm over other enzymes was noticed during the processing of wheat bran, since this preparation was able to deliver up to 18.75 mg 100 g^{-1} of SA within 24 and 48 h of EH. The yield of SA in this study is analogous to that obtained by alkaline-assisted hydrolysis of whole wheat grains [35]. The authors highlighted that SA after FA and dimeric FA is the third most prevalent HCM released from whole wheat grains. However, contradicting results were observed in case of rye bran, where the yield of SA was highest in the sample which underwent EH with Viscozyme L for 24 and 48 h. This time, a comparable amount of SA was found in oat bran hydrolysates after EH with Celluclast 1.5 L, and the highest yield was observed after 48 h of EH. The EH of oat bran with Viscoferm and Viscozyme L regardless of EH duration delivered a lower yield of SA. Similar to this study, the advantage of a developed biorefining process aimed at releasing SA from mustard bran has been demonstrated in the report of Achinivu et al. [36], highlighting better release of SA than that of alkali processed bran.

The small-scale process revealed each enzyme contributed differently to the release of HCMs from bran matrix due to its specificity and activity, though among the enzymes tested, the superiority of multi-enzyme complex Viscozyme L must be highlighted. Overall, a gentler approach ensured up to 215.7% release of the total alkali-extractable FA from rye bran, while 79.5 and 60.3% release can be achieved from wheat and oat, respectively.

3.2. Release of FA from Bran Using Enzyme-Assisted Hydrolysis of 10- and 30-fold Scale-Up Process

The EH at high solids loadings is another important key to the scale-up process for the release of FA from complex matrices. Due to the presence of higher free sugars and other bioactives that can act as inhibitors and therefore interfere with the release of target compounds, the process of scaling is necessary to establish the applicability of developed technology. The increase in solids loading could significantly reduce the efficiency of mass and heat transference, influence the degree of substrate homogeneity, limit the formation of the enzyme-substrate complex by restricting the access of the enzyme to available substrate resulting in lower solubilization of cellulose, hemicellulose, β-glucan, and other non-soluble dietary fiber. There have hitherto been developed various pretreatment strategies regarding the productivity, yield, and the number of steps to enhance the hydrolysis of lignocellulose materials. Over the last years, pilot-scale operational conditions have been proposed for alkaline [37,38], hydrothermal [39], and ultra-fast hydrolysis by supercritical CO_2 pretreatments. More recently, Ferri et al. [6] demonstrated a robust and eco-friendly three-step biorefinery solution for the efficient release of FA. The proposed technology seems to be valid for the industrial-scale production of FA. So far, however, there is limited information on large-scale EH of rye bran, especially at high solids loading, so the current experiment will be focused on scaling-up the EH process accomplished by three hydrolytic enzymes in a single step.

A 10- and 30-fold scale-up was carried out using a bioreactor using the parameters developed for small-scale FA release. The release of FA and other HCMs was analyzed after 48 h of EH, since reduction in the content of both FA isomers was observed for wheat bran, while no significant changes in the release of HCMs were taken place for rye bran during further 24 h of small-scale EH process (Figure 4). Besides, additional energy input would make this process not economically feasible, resulting in a higher price of the final product.

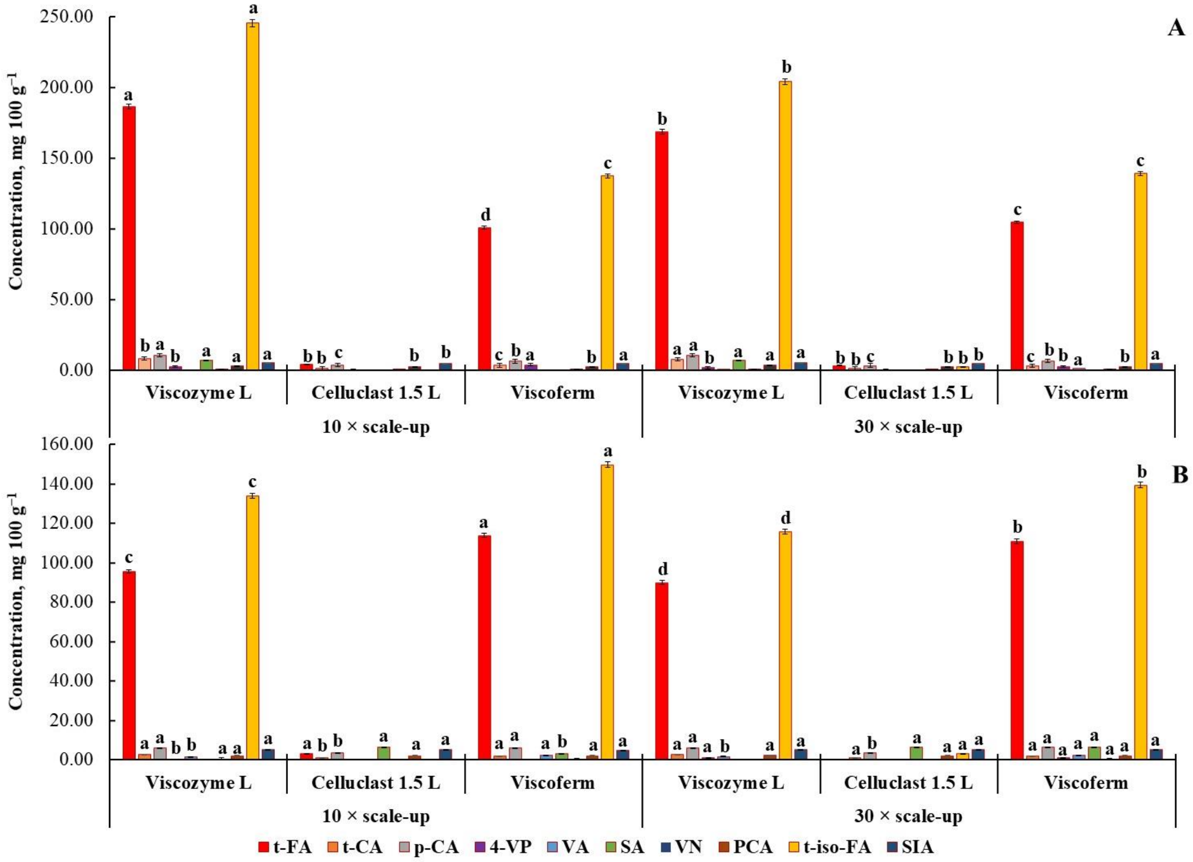

Figure 4. The release of FA and other HCMs after enzymatic hydrolysis of rye (A) and wheat (B) bran for 48 h with three multi-enzyme complexes, i.e., Viscozyme L, Celluclast 1.5 L, and Viscoferm, mg 100 g^{-1} DW. Note: The process was done 10- and 30-fold scaled up process. Values are means ± SD values of triplicates ($n = 3$). Means within the same HCMs with different superscript letters (a, b, c, d) are significantly different at $p < 0.05$.

A 10- and 30-fold scale-up process confirmed the applicability of EH as a bioprocessing type valid for industrial-scale FA production. Similarly, as in small-scale, the advantage of Viscozyme L over other hydrolytic enzymes has been proved scaling up process (Figure 4A).

Chromatographic separation of rye-bran derived HCMs is depicted in Figure 5.

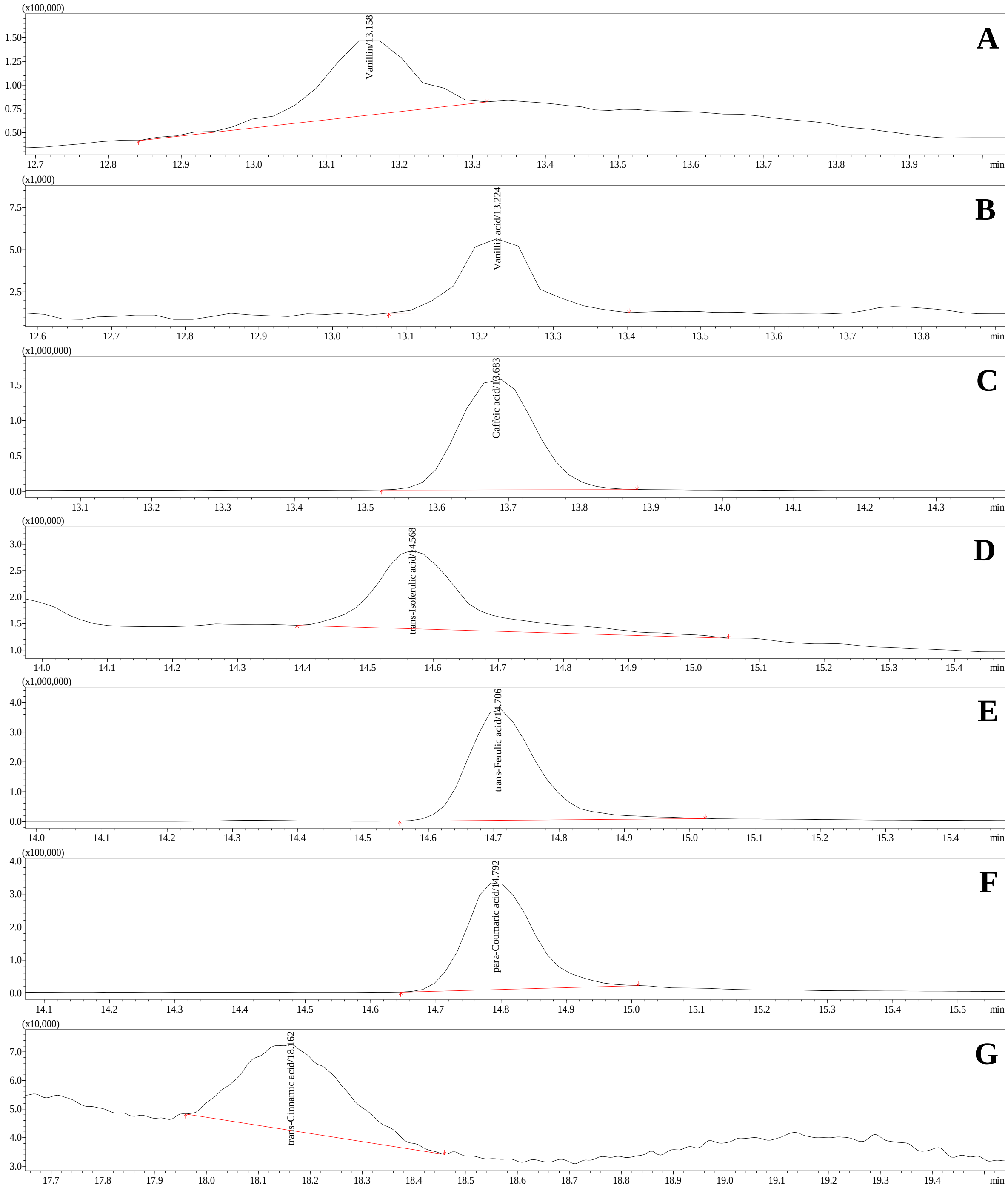

Figure 5. Representative profile of HCMs detected in rye bran hydrolysates: VN (A), VA (B), CA (C), *t*-iso-FA (D), *t*-FA (E), *p*-CA (F), and *t*-CA (G) using a HPLC-ESI-QqQ-MS operating with the multiple reaction monitoring mode.

At 10- and 30-fold scales, up to 431.9 and 372.6 mg of FA (sum of *t*-FA and *t*-iso-FA) can be produced from 100 g of rye bran by the way of EH, respectively. However, as can be seen, the highest concentration of FA was reached at the 10-fold scaled experiment. It is worth noting the amount of FA released at the bioreactor scale was found to be significantly higher than was observed at smaller scales. A plausible explanation for getting a better release of FA was provided by Ferri et al. [6], who posits the bioreactor scale conditions improved EH performance as a result of controlled pH and continuous agitation of the slurry. All this made it attainable to reach a more homogeneous enzymatic reaction and hydrolysis efficiency.

As with small-scale EH, better release of FA from wheat bran was observed during EH with Viscoferm (Figure 4B). The produced amount of FA in both 10- and 30-fold scaled-up experiments was significantly higher than observed for other enzymes. Using proposed conditions 100 g of EH wheat bran could deliver up to 263.6 and 250.2 mg of FA, respectively. As seen, significantly higher yield of *t*-FA and *t*-iso-FA was found for the 10-fold scale-up experiment.

Due to gentler hydrolysis conditions, the scaled-up EH process of rye and wheat bran with Viscozyme and Viscoferm demonstrated up to 217.6 and 91.2% release of the total alkali-extractable FA (sum of *t*-FA and *t*-iso-FA) from rye and wheat bran, respectively.

3.3. Release of FA from Bran Using SFE-CO$_2$ Extraction

The interactions between intracellular phytochemicals and cell walls have a significant influence on the extractability of compounds of interest from complex plant matrices [5], incl. whole grains and their derived fractions. Recently, a couple dozen extractants were investigated and proposed for the industrial-scale production of dietary polyphenols, generally favoring such solvents or their blends as acetone [40], methanol [41], diethyl ether, and ethyl acetate [42], even though they may be harmful to operators and environment. Among the extraction methods documented, superior extractability of phytochemicals from complex matrices was reported for SFE [43]. Due to the GRAS status assigned and relatively low costs, CO$_2$ is the most widely used supercritical fluid suitable for both research purposes and industrial scales. Since neat supercritical fluid CO$_2$ has dissolving propertied close to hexane that is recognized as an excellent solvent for extracting non-polar compounds, the addition of co-solvent could enhance the solubilizing properties of CO$_2$, making it attainable to recover more polar molecules. Due to the process taking place in a closed loop, the online extraction, separation, and analysis of HCMs using SFE-CO$_2$-SFC to MS-TQ make it attainable to reduce both qualitative and quantitative losses of analytes during analytical work.

For comparative purposes of the EH efficiency, the SFE-CO$_2$ extraction coupled to SFC-MS-TQ was applied in this experiment. Since such extraction parameters or their interactions as temperature, pressure, co-solvent concentration, and mode (static and dynamic) could influence the yield compounds of interest, a series of SFE-SFE operational conditions were investigated in a lab-scale Nexera UC SFE-SFC-LC-MS-QT apparatus (Table 3).

In the first test runs 1–4, the influence of extraction temperature over the range of 40–60 °C was investigated, while pressure, co-solvent concentration (modifier), and extraction parameters were kept constant at 10 MPa, 10% EtOH, and 3:3 min, respectively (Figure 6). The selected temperature range was based on a previous report [44]. As expected, the yield of FA and other HCMs increased with the increase in extraction temperature, while, rising the temperature over 50 °C, the amount of *t*-FA decreased significantly ($p < 0.05$), and the observed value was even lower than that obtained at 45 °C (Figure 6A). The results are consistent with those of [45,46], indicating that the vast majority of phenolics and antioxidants present in grape seeds and cranberry bush berries and pomace were extracted using a temperature range of 44–46 °C. A plausible explanation for getting better release of FA has been given by Ameer et al. [43], pointing out that elevated temperature after plant matrix is wetted allows reducing surface tension and rising solvent cavitation that accelerate dissolution of analyte in the solvent.

It is worth noting the presence of *t*-iso-FA was found only when rye bran samples were subjected to extraction at 50 °C temperature. Therefore, an extraction temperature of 50 °C was used for subsequent extractions. The third prevalent compound extracted from rye bran samples by means of SFE-CO$_2$ was VA. The yield of this HCM fluctuated in the range of 133.2–166.4 mg 100 g^{-1}. It should be admitted that the value of VA was found to be significantly higher than yielded during EH, indicating possible decarboxylation process of free VA caused by elevated temperature during hydrolysate autoclaving. The presence of 4-VG as the primary degradation product of VA is also confirmed by [47].

To evaluate the effect of pressure on the extractability of FA from rye bran matrix by SFE-CO$_2$, the experiments (5–8) were carried out using extraction pressure within the range of 10–25 MPa at a constant temperature (50 °C), concentration of modifier (10%), and the length of static to dynamic mode (3:3 min).

As seen (Figure 6B), the yield of *t*-FA was significantly higher when rye bran samples were subjected to 25 and 15 MPa extraction pressures. However, when extracting at 25 MPa, the presence of *t*-iso-FA was not detected, revealing higher susceptibility of *t*-iso-FA to degradation by elevated pressure than *t*-FA. Although, when calculating the total amount of these two HCMs, the obvious advantage of using 15 MPa pressure among all other pressures is noticed. Considering the yield of other HCMs, it is seen that there were no significant differences in the content of CA, *p*-CA, and VN, therefore the pressure of 15 MPa was selected for further extractions to minimize wear of the machine parts.

To study the influence of co-solvent concentration on the extractability of FA from rye bran, the experiments in the next series of runs (9–12) were conducted. The addition of EtOH in the range of 7.5–20% along with the main supercritical CO_2 extraction fluid was used in this experiment, while moderate operating conditions such as temperature – 50 °C, pressure – 15 MPa, and the length of static to dynamic mode – 3:3 min were maintained constant (Figure 6C). Since *t*-FA and *t*-iso-FA present both methoxy and hydroxy substituents, and also to keep the SFE-CO_2 extraction "green", the polar EtOH has been selected as a co-solvent. The results showed that almost equal yields of *t*-FA could be reached with the addition of polar EtOH to the CO_2 extraction fluid in the range of 7.5–10%, though, the increase of the extraction solvent polarity and elevated viscosity resulted in a marked reduction of *t*-FA solubility and recovery. The yield of *t*-iso-FA in the fraction obtained with the use of 10% EtOH as a co-solvent was found to be the highest. In turn, no presence of *t*-iso-FA was found in the extracts obtained with the addition of 15 and 20% EtOH. The results are consistent with data obtained in an earlier study [48], highlighting that, due to relatively low toxicity and potential applications of obtained extracts in food, cosmetic and pharmaceutical products this solvent has advantages over others. The total FA content was relatively higher by performing the extraction with the addition of 10% EtOH, therefore this concentration has been selected for subsequent extractions.

In the final set of experiments (13–17), when optimal values for temperature, pressure, and concentration of co-solvent for extracting of FA from bran matrix have been elucidated, the influence of extraction mode, i.e., static and dynamic and its length was investigated. As it turned out, the type of extraction has also a crucial role that could affect the yield of FA (Figure 6D). The lowest yield of *t*-FA was obtained when the extraction was done by 5 min static and 1 min dynamic mode, however, the amount of *t*-iso-FA in the extracts was the highest. To find a compromise between the yield of both *t*-FA and *t*-iso-FA, the extraction of HCMs was done using a 4 min static and 2 min dynamic mode. As can be seen, a 89.9% higher yield of *t*-FA has reached, while no presence of *t*-iso-FA was detected in the extracts obtained using this mode. A satisfactory yield of *t*-FA and *t*-iso-FA was reached only after shifting from 5 min static extraction mode to 5 min dynamic (static to dynamic ratio – 1:5). A credible explanation for getting better extractability of compounds applying longer dynamic mode has been given by Luque de Castro et al. [49], pointing out continuous exposure of analyte to the clean extraction solvent could enhance of displacement of the analyte partitioning equilibrium to the mobile phase.

To summarize, the introduced SFE-SFC approach is another eco-friendly alternative suitable for industrial-scale production of FA. The extractability of FA (sum of *t*-FA and *t*-iso-FA) by means of CO_2 and 10% EtOH as a co-solvent, 15 MPa pressure, 50 °C temperature, and the length of the static 1 min and dynamic mode 5 min delivered an increase of obtained FA by 164.0% in rye, 69.9% in wheat and 329.9% in oat bran in comparison to FA obtained after alkaline-assisted hydrolysis (Figure 6E).

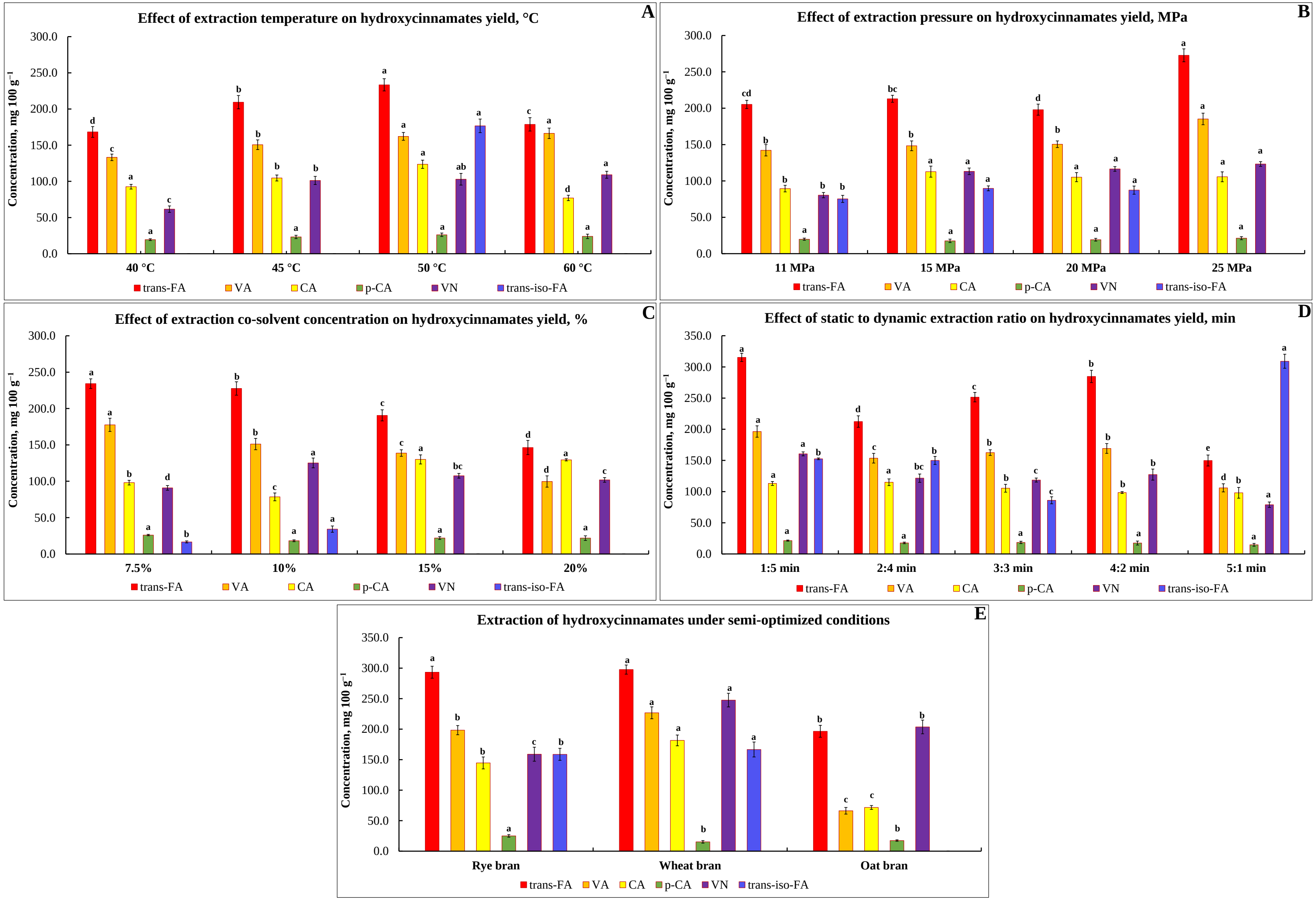

Figure 6. The release, isolation, and analysis of rye bran derived HCMs using variable (A, B, C, D) and semi-optimized SFE-CO_2 extraction conditions (E) for extraction of HCMs from bran samples, mg 100 g^{-1} DW. Note: Values are means ± SD values of triplicates ($n = 3$). Means within the same HCM with different superscript letters (a, b, c, and d) are significantly different at $p < 0.05$.

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3.4. Release of Mono- and Disaccharides from Bran Using Enzyme-Assisted Hydrolysis

According to Novozymes®, hydrolytic enzymes selected in this study contain a wide range of carbohydrases, i.e., xylanases, cellulases, arabanases, glucanase, and hemicellulose where the end products of synergistic action could be glucose, arabinose, mannose, galactose, and xylose. It has been demonstrated that FA both in grains and grain-derived fractions is presented in covalently cross-linked form with arabinofuranosidase residues in arabinoxylans, while continuous EH with lignocellulose-degrading enzymes results in solubilization of water-insoluble and depolymerization of water-soluble arabinoxylans with the simultaneous release of FA [5]. This statement has been reinforced by Shin et al. [50], revealing a strong correlation between the release of FA and the yield of reducing sugars during EH of corn fiber.

Previous experiments revealed the ability of selected hydrolytic enzymes to release bound FA forms from rye, wheat, and oat bran matrix within a single process step of EH. Therefore, the next experiment will be aimed at assessing the yield of mono- and disaccharides over 72 h of EH. A detailed profile of sugars after small-scale EH is depicted in Table 5. Glucose, xylose, arabinose, and fructose were the major products released from rye, wheat, and oat bran (Supplementary Figure S2), though the amount of sugars varied depending on the hydrolytic enzyme applied and length of EH. The presence of galactose was also confirmed in bran hydrolysates, though further work with direct enzymes injections revealed the availability of this sugar as part of commercial enzymes preparations.

The action of α-L-arabinofuranosidase has resulted in the cleavage of non-reducing end of terminal α-L-arabinofuranoside residues in α-L-arabinosides, and therefore the presence of feruloylated arabinose monomers at the initial stage of EH was revealed. Viscoferm displayed maximum hydrolytic performance after 12 h of EH since the highest yield of arabinose in rye bran hydrolysates made up 7.9 g 1000 mL^{-1}. The obtained results are consistent with data by Gama et al. [51], indicating that arabinofuranosidase is more active at the initial stage of EH since, by the action of this enzyme, smaller xylan polysaccharides are formed that contain no side chains of arabinose residues, thereby ensuring the xylan area is more accessible to enzymes involved in splitting of xylosidic bonds with the simultaneous release of xylooligosaccharides along with xylose monomers. However, it is worth noting that at the end of 72 h a 28.4% decrease of this monosaccharide was noticed. A similar trend of arabinose decrease has been found in rye bran hydrolysates subjected to EH with Viscozyme L, where up to 40.6% of arabinose loss was observed after 72 h of EH. Opposite results were obtained during EH of wheat bran samples with Viscoferm, where up 150.5% increase of arabinose was observed at the end of 72 h. Considerable fluctuations in this monomer content were detected during EH of wheat bran with Viscozyme L, the maximum peak of which was marked upon 48 h of EH. While additional 24 h hydrolysis resulted in a 44.4% reduction of this monomer. A credible explanation of this phenomenon has been given by Xin et al. [52], pointing out that continuation of the hydrolytic activity of α-L-arabinofuranosidase can cause extensive loss of free arabinose.

Xylose is a sugar monomer that can be released during EH through the action of xylanolytic enzymes that are capable of depolymerizing xylosidic bonds present in the xylan backbone chain. The highest content of xylose monomers was observed after 48 h of EH with Viscozyme L. Up to 12.8 and 10.6 g 1000 mL^{-1} of xylose was released from rye and wheat bran samples, demonstrating superior hydrolytic activity of this enzyme over other applied enzymes. Contrary results were reported by Bautista-Expósito et al. [53], demonstrating the superior xylanolytic performance of Viscoferm since the amount of xylose released was 7.4-fold higher than if using Viscozyme L.

According to Novozymes®, the main activity of hydrolytic enzymes selected is being the glycosidic, in addition to arabinose and xylose content, the efficiency of hydrolysis has been estimated by measuring the amount of glucose in hydrolysates. The results of glucose content analysis show values ranging between 5.8 to 59.4 g 1000 mL^{-1} for rye, wheat, and oat bran hydrolysates, with oat bran having the highest content, while wheat bran the lowest. A higher amount of glucose in oat bran hydrolysates is explained by the presence of the relatively higher amount of β-glucan, which alongside to cellulose by the action of endo-1,4-β-D-glucanase release glucose monomers [54]. The maximum yield of glucose was released after 48 h of EH with Viscozyme L. The same observation has been made by Bautista-Expósito et al. [53], highlighting that Viscozyme L over 13 other commercial enzymes tested was the most efficient, able to release the highest amount glucose monomers from wheat bran polysaccharides. EH with Viscozyme L for 48 h has led to an up 76.5, 81.5, and 5.1% increase in glucose content as compared to the initial level obtained after 12 h of rye, wheat, and oat bran EH, respectively. The highest release of glucose using Viscoferm was reached only passing 72 h of EH, corresponding to 83.7 and 62.9% increase for rye and oat bran hydrolysates, respectively, while continuous EH of wheat bran samples during 72 h has failed to release higher amount of glucose.

Table 5. The release of mono- and disaccharides during 72 h enzymatic hydrolysis of bran samples using three multi-enzyme complexes, i.e., Viscozyme L, Celluclast 1.5 L, and Viscoferm, g 1000 mL^{-1} of bran hydrolysate.

Enzyme	**Viscozyme L**				**Celluclast 1.5 L**				**Viscoferm**			
Time, h	**12**	**24**	**48**	**72**	**12**	**24**	**48**	**72**	**12**	**24**	**48**	**72**
Carbo-hydrate	**Rye bran**											
Xyl	6.78 ± 0.61	9.43 ± 0.55	12.87 ± 0.04	9.67 ± 0.05	10.64 ± 0.65	12.30 ± 0.96	12.77 ± 0.65	11.11 ± 0.41	8.89 ± 0.47	9.91 ± 0.52	10.80 ± 0.24	10.87 ± 0.65
Ara	5.32 ± 0.52	4.22 ± 0.48	5.14 ± 0.03	3.16 ± 0.04	2.52 ± 0.08	2.74 ± 0.34	2.53 ± 0.19	2.22 ± 0.25	7.98 ± 0.14	7.39 ± 0.96	4.59 ± 0.42	5.71 ± 0.43
Fru	2.50 ± 0.32	2.97 ± 0.33	4.41 ± 0.09	1.58 ± 0.01	2.65 ± 0.07	2.91 ± 0.05	3.07 ± 0.13	2.51 ± 0.21	2.24 ± 0.17	3.06 ± 0.45	2.18 ± 0.09	2.88 ± 0.14
Glu	22.22 ± 0.43	34.13 ± 0.94	39.88 ± 0.85	31.54 ± 0.98	11.50 ± 0.21	13.44 ± 0.75	13.27 ± 0.87	11.84 ± 0.56	10.81 ± 0.42	10.85 ± 0.85	10.75 ± 0.12	19.86 ± 0.74
Suc	0.19 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.37 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mal	0.83 ± 0.09	1.36 ± 0.04	1.34 ± 0.01	1.45 ± 0.01	0.57 ± 0.02	1.34 ± 0.07	1.37 ± 0.08	1.46 ± 0.04	0.50 ± 0.09	1.79 ± 0.14	1.72 ± 0.05	1.74 ± 0.07
Total	37.84 ± 1.98	52.12 ± 2.34	63.64 ± 1.02	47.40 ± 1.09	27.88 ± 1.03	32.74 ± 2.17	33.00 ± 1.92	29.13 ± 1.47	30.79 ± 1.36	33.00 ± 2.92	30.04 ± 0.83	41.06 ± 2.03
Carbo-hydrate	**Wheat bran**											
Xyl	6.36 ± 0.65	8.17 ± 0.15	10.61 ± 0.45	8.90 ± 0.64	8.64 ± 0.43	10.68 ± 0.21	12.07 ± 0.23	9.47 ± 0.23	1.38 ± 0.11	8.60 ± 0.41	9.74 ± 0.46	10.28 ± 0.55
Ara	1.89 ± 0.15	3.10 ± 0.23	5.11 ± 0.21	2.84 ± 0.35	2.11 ± 0.21	3.59 ± 0.15	4.11 ± 0.14	1.96 ± 0.14	1.98 ± 0.08	4.03 ± 0.39	4.31 ± 0.25	4.96 ± 0.25
Fru	1.02 ± 0.12	1.89 ± 0.09	4.02 ± 0.11	1.48 ± 0.21	2.51 ± 0.12	3.44 ± 0.23	3.70 ± 0.21	2.63 ± 0.11	0.86 ± 0.04	1.85 ± 0.15	2.47 ± 0.13	2.87 ± 0.36
Glu	13.84 ± 0.43	18.99 ± 0.85	25.12 ± 0.13	18.67 ± 0.34	5.88 ± 0.14	6.18 ± 0.25	6.74 ± 0.28	7.44 ± 0.32	17.26 ± 0.36	6.59 ± 0.09	7.43 ± 0.12	15.98 ± 0.74
Suc	1.20 ± 0.11	0.64 ± 0.09	0.40 ± 0.02	0.13 ± 0.05	0.55 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mal	1.05 ± 0.09	1.56 ± 0.08	1.65 ± 0.09	1.87 ± 0.03	1.09 ± 0.08	1.11 ± 0.065	1.28 ± 0.02	1.28 ± 0.08	0.45 ± 0.12	1.31 ± 0.13	1.44 ± 0.11	1.65 ± 0.09
Total	25.36 ± 1.55	34.34 ± 1.49	46.90 ± 1.01	33.88 ± 1.62	20.78 ± 1.10	24.99 ± 0.91	27.90 ± 0.88	22.79 ± 0.88	21.93 ± 0.71	22.37 ± 1.17	25.39 ± 1.07	35.74 ± 1.99
Carbo-hydrate	**Oat bran**											
Xyl	1.22 ± 0.11	1.39 ± 0.12	1.64 ± 0.14	1.87 ± 0.07	1.64 ± 0.07	3.15 ± 0.11	1.49 ± 0.08	1.86 ± 0.03	1.41 ± 0.03	1.39 ± 0.05	1.58 ± 0.15	1.84 ± 0.09
Ara	1.08 ± 0.09	1.17 ± 0.08	1.10 ± 0.08	1.57 ± 0.14	0.00 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.28 ± 0.08	1.13 ± 0.06	1.10 ± 0.06	1.28 ± 0.04
Fru	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.72 ± 0.05	0.79 ± 0.13	0.89 ± 0.08	0.97 ± 0.15
Glu	56.58 ± 1.90	62.26 ± 1.57	59.46 ± 1.23	55.49 ± 2.11	7.00 ± 0.32	10.40 ± 0.57	12.64 ± 0.36	17.14 ± 0.41	13.90 ± 0.63	16.20 ± 0.93	20.35 ± 0.12	22.64 ± 0.55
Suc	0.00 ± 0.0	0.36 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.10 ± 0.13	1.40 ± 0.08	1.60 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mal	0.59 ± 0.12	0.96 ± 0.03	0.96 ± 0.07	0.98 ± 0.09	0.31 ± 0.07	0.53 ± 0.04	0.52 ± 0.08	0.56 ± 0.06	0.42 ± 0.09	1.54 ± 0.07	2.21 ± 0.13	2.07 ± 0.08
Total	59.47 ± 2.22	66.14 ± 1.88	63.16 ± 1.52	59.91 ± 2.41	8.96 ± 0.54	15.18 ± 0.85	16.05 ± 0.60	21.16 ± 0.62	17.73 ± 0.88	21.05 ± 1.24	26.12 ± 0.54	28.81 ± 0.91

Note: Values are means ± SD values of three replicates (*n* = 3). Xyl - Xylose, Ara – Arabinose, Fru – Fructose, Glu – Glucose, Suc – Sucrose, Mal – Maltose.

3.5. Release of Mono- and Disaccharides from Bran Using Enzyme-Assisted Hydrolysis of 10- and 30-fold Scale-Up Process

A 10- and 30-fold scale-up process was carried out using a bioreactor. The release of individual sugars has been analyzed after 48 h of EH, since during further 24 h of the small-scale process due to the continuation in the hydrolytic activity of α-L-arabinofuranosidase present in Viscozyme L an extensive reduction in arabinose content was observed in all bran hydrolysates. A scale-up process revealed a similar trend of individual sugars increasing after 48 h of EH. The advantage of Viscoferm over other hydrolytic enzymes has been proven in both scale-up experiments at high solids load, though the amount released varied depending on the type of sugar analyzed. As is seen, the amount of arabinose in bran hydrolysates was found in the range from 1.2 to 9.0 and from 0.9 to 7.1 g 1000 mL^{-1} for 10- and 30-fold scale-up experiments, respectively. The highest arabinose content has been observed in rye bran hydrolysates underwent EH with Viscoferm and Viscozyme L, though it was 97.2 and 37.7% higher than observed during small-scale EH, respectively (Figure 7A and 7D). As shown, the highest concentration of xylose monomer in both scale-up experiments was reached after EH with Celluclast L, with rye bran having the highest content (Figure 7A and 7D), while oat bran the lowest (Figure 7C and 7F).

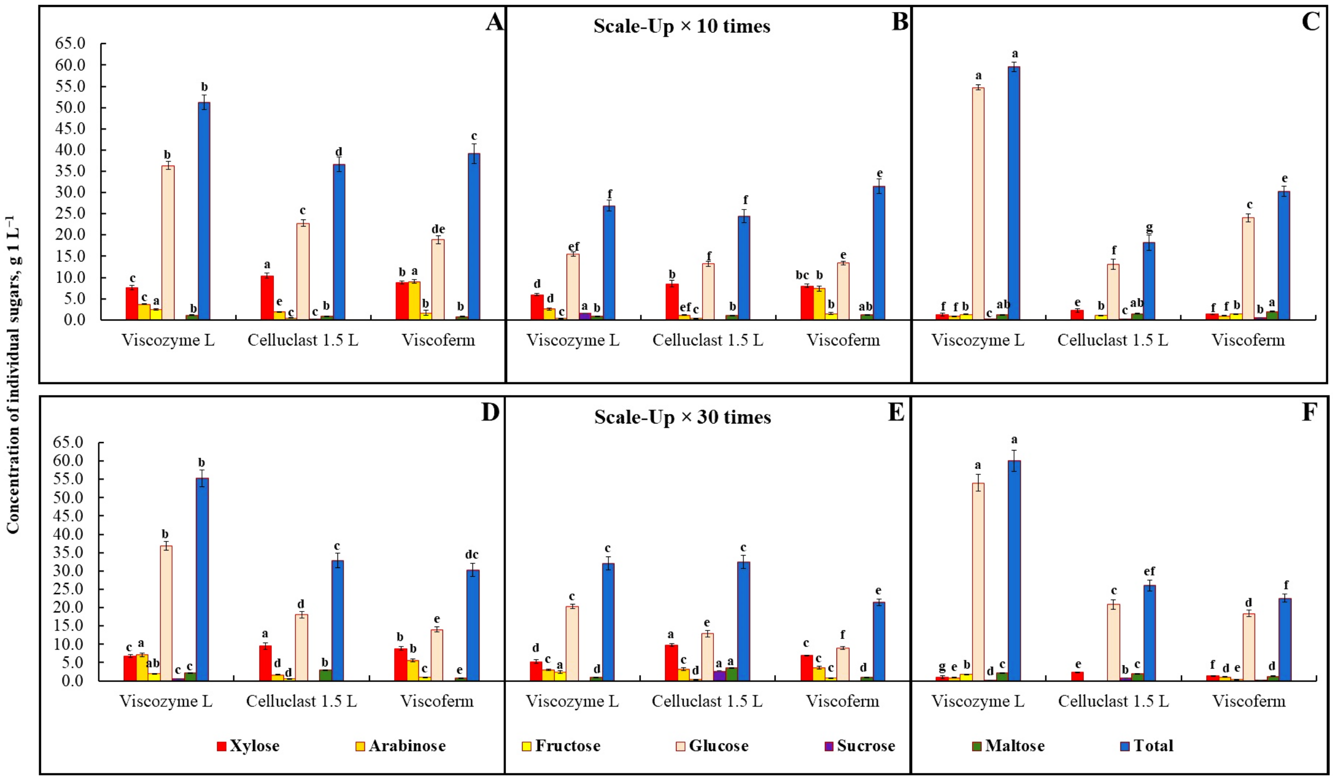

Figure 7. The release of mono- and disaccharides after enzymatic hydrolysis of rye (A, D), wheat (B, E), and oat (C, F) bran samples for 48 h with three multi-enzyme complexes, i.e., Viscozyme L, Celluclast 1.5 L, and Viscoferm, g 1000 mL^{-1} of hydrolysate. Note: The process was done 10- and 30-fold scaled up process conditions. Note: Values are means ± SD values of triplicates (n = 3). Means within the same sugar with different superscript letters (a, b, c, d) are significantly different at $p < 0.05$.

The amount of xylose released was 18.6% (10-fold) and 25.5% (30-fold) lower than observed at small-scale rye bran EH. Viscozyme L demonstrated the lowest hydrolytic activity at high solids load in relation to xylose yield, which is possibly associated with high susceptibility of xylanase to external factors such as high content of other sugars monomers or availability of free bioactives that have exhibited inhibitory activities. However, assessing the glucose yield obtained in the scaled-up EH process, the superiority of Viscozyme L over other enzymes was highlighted for rye and wheat bran in comparison with small-scale EH experiment. The amounts of glucose obtained at both 10- and 30-fold scale-up conditions were 7.8–8.8%, 19.0–38.3%, and 8.0–9.1% lower than released during small-scale EH of rye, wheat, and oat, respectively.

Evaluation of the total amount of sugars revealed lower release in both 10- and 30-fold scaled experiments than acquired during small-scale EH process, however, the proposed EH conditions are still valid, since the 100 g of enzymatically hydrolyzed rye, wheat, and oat could deliver up to 55.3, 32.5, and 60.0 g of sugars, respectively. In general, the highest total sugars yield was obtained during EH of bran with Viscozyme L, except for wheat (10-fold scale), where Viscoferm demonstrated relatively better hydrolytic performance. In the context of the non-waste technology, the mono- and disaccharides released have great potential to be used in bioethanol fuel production, however, a proper purification process needs to be developed [37,55].

3.6. Recovery of FA from Bran Hydrolysates

In this experiment, the hydrolysates of rye bran acquired after EH with Viscozyme L were subjected to SPE purification using polymeric reversed-phase Strata X column (30 mg 3 mL^{-1}). The selection of the sorbent was based on availability, effectiveness, and simplicity of use, since no specific activation, i.e., thermal, acidic, or base aside from conditioning with either MeOH or EtOH are required [56].

The solubility of FA can vary depending on the solvent and the concentration used. In the earlier report of Couteau et al. [57], positive desorption of FA from polyvinylpolypyrrolidone (PVPP) with a purity of about 62% was achieved by 96% EtOH, while later on, 97.4% recovery of FA has been demonstrated by 60% EtOH [58]. The contradiction in results on recovery of FA using different concentrations of alcohol promoted the design of this experiment to study the recovery and purity of FA using MeOH and the less toxic EtOH and their aqueous solutions as elution solvents. The purity of FA in collected fractions was studied chromatographically by assessing the presence of side HCMs and mono- and disaccharides.

The results on FA recovery by means of MeOH and their aqueous solutions revealed increasing desorption of both *t*-FA and *t*-iso-FA in a MeOH concentration-dependent manner (Figure 8A). Up to 67.2 and 80.1% recovery of *t*-FA and *t*-iso-FA from rye bran hydrolysates was achieved by means of acidified 80% MeOH (2% formic acid, *v*/*v*), respectively. The overall purity of FA in the fraction collected was 82.7%. Application of acidified 90% MeOH brought a rise in the recovery of *t*-FA and *t*-iso-FA, since 70.6 and 83.6% of these HCMs were observed in the collected phase, but the relative purity of FA was 83.4%. To improve the purity of FA, an additional wash-up step with 5% MeOH was done after FA-rich hydrolysates were passed through the column and afterward eluted by 90% MeOH. This approach has brought up to 90.1% purity of FA with a relative recovery of 71.6%.

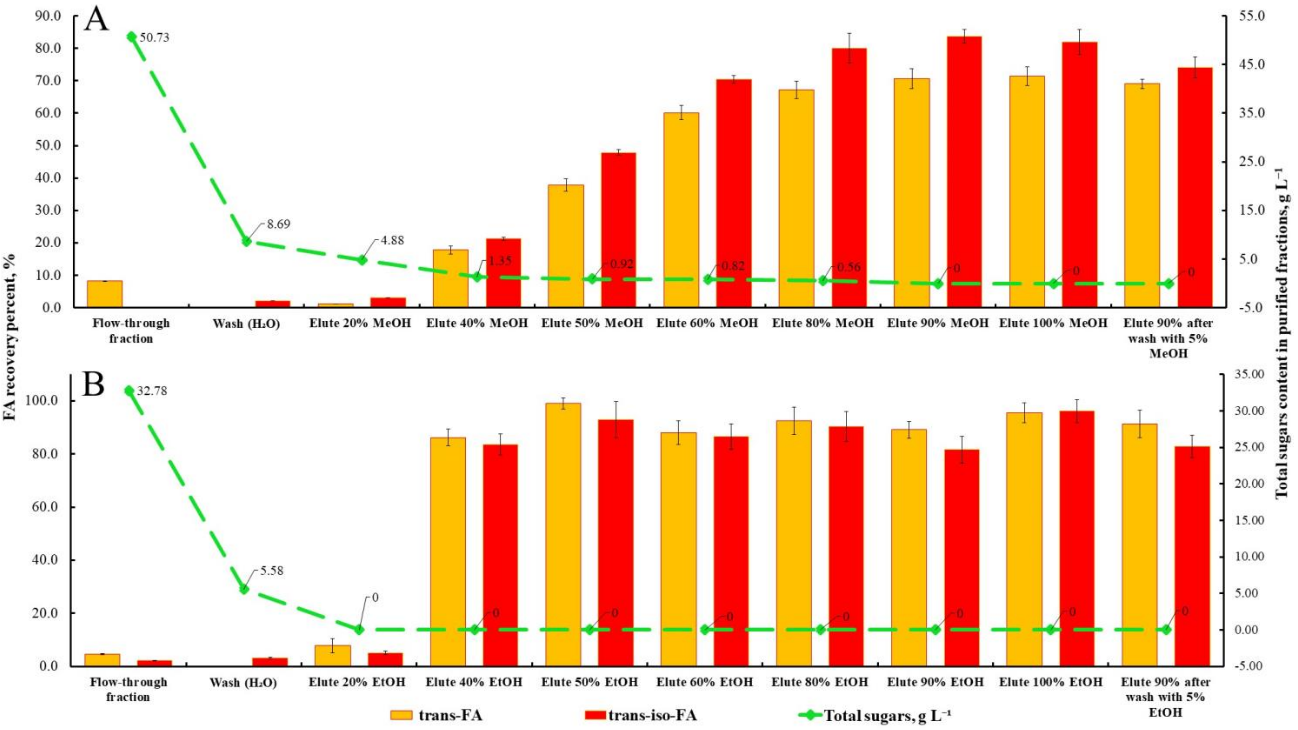

Figure 8. Recovery percentage of *t*-FA and *t*-iso-FA following solid-phase extraction by Strata X column using MeOH (A) and EtOH (B) and their aqueous solutions as eluent. Note: Values are means ± SD values of triplicates (*n* = 2). Green line on the plot indicates the residual concentration of sugars, g L^{-1} in fractions collected.

Satisfactory results on recovery of FA from rye bran hydrolysates were obtained with the application of EtOH as eluent (Figure 8B). At the first step of purification, the 20 and 40% EtOH were used to check the validity of these solvents for desorbing of FA retained on the stationary phase of Strata X column. As revealed, the selected concentrations of EtOH were found be to less effective in desorbing either *t*-FA or *t*-iso-FA from the polymer selected. However, the recovery of these two compounds was 67.5 and 97.5% more effective than achieved by MeOH, respectively. An increase in EtOH concentration up to 50% resulted in a rise in the recovery of *t*-FA and *t*-iso-FA up to 99.1 and 92.9%. Application of this eluting agent ensured 94.0% purity of FA obtained.

3.7. *Fatty Acid Composition of Brown Lipophilic Substance*

Buranov et al. [59] indicated in a report that the alkaline-derived hydrolysates of wheat and corn bran was contained a brown lipophilic substance that made the process of FA purification challenging. The presence of the brown substance has also been mentioned in a report by Salgado et al. [56], noting that it can be easily precipitated by adding 30% EtOH to the alkali extract, followed by centrifugation. However, neither of the reports specified the composition of the waxy substance observed, therefore in the further section characterization of the lipophilic fraction will be given based on fatty acid (FTA) content (Table 6).

The results demonstrated significant variations in FTA percentage distribution between hydrolysis methods used. The abundance of unsaturated FTA was revealed for lipids obtained after EH of bran, making up 77–81% of the total FTA (in relative percentage), while 43–61% of unsaturated FTA were observed in lipids of alkaline-assisted hydrolysates. Linoleic acid (C18:2), oleic acid (C18:1), and palmitic acid (C16:0) contributed the most to the total amount of FTA, corresponding to about 93.0 to 98.0%. The results on relative percentage of FTA in rye and wheat bran are consistent with data obtained by Cardoso et al. [60], noting that C18:2, followed by C18:1, C16:0, and to a lesser extent C18:3 are the most abundant FTA found. The amount of SFA in the lipid fraction of oat bran lipids varied in the range of 4.1–9.6%, while PUFA was in the range of 39.4–46.3%. C18:1 was found to be the most dominant fatty acid, the percentage of which was in the range of 13.1–29.0%. A similar contribution of SFA to the total amount of FTA was reported for oat lipids after alkaline hydrolysis, followed by methylation in boron trifluoride-methanol [61]. A higher amount of PUFA was found in rye-derived lipids upon subjecting bran samples to EH with Celluclast 1.5 L and alkaline-assisted hydrolysis since the concentration was 16.0–23.7% and 18.7% higher than that observed in oat bran-derived lipids, respectively.

The total content of FTA in bran-derived lipids has been found to be hydrolysis method dependent that varied in the range of 21.4 ± 1.0 to 101.4 ± 6.0 g 100 mL^{-1}. The advantage of Celluclast L over other enzymes used in relation to FTA release from wheat bran samples was observed. The amount of FTA in wheat-derived lipids was found 289.9% higher than obtained by alkaline hydrolysis. Notably weaker, but still effective release of FTA from rye and wheat bran matrix was observed upon EH with Viscozyme L. EH of rye and wheat bran in comparison to alkaline-assisted hydrolysis has ensured the release of 68.4 ± 7.8 and 51.3. ± 2.4 g 100 mL^{-1} of FTA, which is 160.9 and 87.8% more than alkaline hydrolysis does. The EH with Viscoferm in comparison to alkaline hydrolysis was able to release 25.4 and 18.8% more FTA from oat and wheat bran matrix, while no obvious effect was observed for rye bran.

In general, considering Viscozyme L and Viscoferm have higher declared xylanolytic activity than cellulolytic, which is also confirmed by Wikiera et al. [62], it can be assumed that the degradation of cellulose composed cell-wall membranes by means of cellulolytic enzymes is more important for the efficient release of FTA than by xylanolytic enzymes.

Table 6. Fatty acid composition of bran hydrolysates according to the hydrolysis method.

Type of Bran	HM	Fatty acid, % *w/v*							
		SFA		MUFA			PUFA		
		C16:0	C18:0	C18:1n9c	C18:1n9t	C20:1n9c	C18:2n6c	C18:3n3c	C20:3n3c
Rye	KOH (10% in MeOH)	5.46 ± 0.06	0.33 ± 0.00	4.18 ± 0.19	nd	0.06 ± 0.01	14.92 ± 0.13	1.27 ± 0.04	< LOQ
	Viscozyme L	14.24 ± 1.16	0.89 ± 0.13	12.13 ± 1.36	nd	2.46 ± 0.74	36.10 ± 1.24	2.62 ± 0.26	nd
	Celluclast 1.5 L	7.97 ± 0.61	0.37 ± 0.09	5.88 ± 0.44	nd	nd	22.72 ± 0.26	1.58 ± 0.10	nd
	Viscoferm	4.39 ± 0.10	0.31 ± 0.02	3.22 ± 0.34	nd	nd	12.92 ± 0.36	0.59 ± 0.20	nd
Wheat	KOH (10% in MeOH)	5.62 ± 0.08	0.38 ± 0.02	5.40 ± 0.08	nd	0.01 ± 0.0 0	14.80 ± 0.68	1.10 ± 0.07	0.02 ± 0.01
	Viscozyme L	10.19 ± 0.29	0.67 ± 0.06	10.86 ± 0.91	0.05 ± 0.02	0.23 ± 0.05	27.00 ± 0.99	2.34 ± 0.14	nd
	Celluclast 1.5 L	22.88 ± 0.39	1.22 ± 0.01	21.06 ± 0.31	nd	nd	57.09 ± 0.42	4.34 ± 0.06	nd
	Viscoferm	6.80 ± 0.30	0.45 ± 0.12	6.64 ± 0.30	nd	nd	17.87 ± 0.40	0.72 ± 0.03	nd
Oat	KOH (10% in MeOH)	7.12 ± 0.05	0.54 ± 0.01	13.15 ± 0.66	nd	0.12 ± 0.01	15.30 ± 0.35	0.54 ± 0.10	nd
	Viscozyme L	12.37 ± 1.35	1.01 ± 0.08	29.02 ± 3.75	nd	0.43 ± 0.05	28.14 ± 4.49	1.10 ± 0.19	nd
	Celluclast 1.5 L	12.20 ± 0.25	0.75 ± 0.15	27.88 ± 0.27	nd	nd	25.89 ± 0.70	0.66 ± 0.28	nd
	Viscoferm	8.32 ± 0.01	0.66 ± 0.08	18.62 ± 0.31	nd	nd	18.35 ± 0.38	0.19 ± 0.10	nd

Note: HM – hydrolysis method; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; nd – not detected; < LOQ – below limit of detection.

4. Conclusions

The present study was undertaken to establish the hydrolytic ability of lignocellulose-degrading enzymes and release bound ferulic acid (FA) during enzymatic hydrolysis (EH) bran. For these purposes, three types of bran, i.e., rye, wheat, and oat were subjected to EH. The release of mono- and disaccharides, FA, and other hydroxycinnamates (HCMs) was monitored throughout the entire process of EH. For comparative purposes, the efficiency of EH in releasing FA along with alkaline-assisted hydrolysis was assessed by an online SFE-SFC-MS extraction approach. The process of small-scale EH revealed that each enzyme, due to its specificity and activity, contributed differently to the release of HCMs from bran matrix, though, among the enzymes tested, the advantage of Viscozyme L was highlighted. Overall, a gentler approach demonstrated up to 215.7% release of the total alkali-extractable FA (sum of *t*-FA and *t*-iso-FA) from rye bran, while 79.5, and 60.3% was released from wheat and oat bran, respectively. Favorable EH conditions during the scale-up process ensured comparable to small-scale process release of FA from rye and wheat bran. Up to 217.6 and 91.2% release of the total alkali-extractable FA was achieved from rye and wheat bran performing EH with Viscozyme L and Viscoferm, respectively. The introduced SFE-CO_2-EtOH extraction approach revealed a significantly higher release of FA, though the extractability varied depending on the parameters used. Extraction by CO_2 and 10% EtOH as a co-solvent, 15 MPa pressure, 50 °C temperature, and the length of the static and dynamic mode of 1 and 5 min was able to deliver by 164.0, 69.9, and 329.9% higher amount of FA than was obtained after alkaline-assisted hydrolysis of rye, wheat, and oat bran, respectively. The experimental results on recovery and purification demonstrated the applicability of styrene-divinylbenzene-based reversed-phase Strata X column for removal of degradation product from bran hydrolysates. In the purified fraction, the absence of residual HCMs and carbohydrates has been confirmed, making recovered FA of 94.0% purity.

The scale-up process confirmed the validity of the technology for industrial-scale production, which altogether could be treated as a green alternative and could represent the future of sustainable FA production.

Author Contributions

Conceptualization, V.R. and K.J.-R.; data curation, V.R.; formal analysis, V.R., J.K. and F.V.; funding acquisition, V.R.; investigation, V.R.; methodology, V.R., K.J.-R. and J.K.; project administration, K.J.-R.; resources, G.L. and A.V.; software, V.R.; visualization, V.R.; writing—original draft, V.R.; writing—review and editing, V.R. and D.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data sets and analysis of current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

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

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