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Polyphenols and Resveratrol from Discarded Leaf Biomass of Grapevine (*Vitis* sp.): Effect of Cultivar and Viticultural Practices in Estonia

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Received: 5 August 2020; Accepted: 3 September 2020; Published: 4 September 2020

Abstract: Grapevine leaves are a major by-product of viticulture practices derived from the leaf-removal from the fruit cluster zone in all vine growing regions. These leaves can be a valuable source of antioxidants to be used in pharmaceuticals or other health-related products. In this study, the leaves of grapevine cultivars were analysed by ultra-high performance liquid chromatograph-diode array detector () for the total polyphenols (TPC) and resveratrol affected by cultivar, leaf-removal time and viticultural practice. The effect of cultivar varied yearly, European grapevine cv. 'Regent' had increased TPC and resveratrol in comparison to 'Boskoop's Glory', 'Rondo' and 'Solaris' in 2017, but 'Solaris' in 2018. TPC (1213–1841 mg 100 g^{-1}) and resveratrol (1.061 mg 100 g^{-1}) were higher in leaves of interspecific hybrid cvs. 'Zilga' and 'Hasansky Sladky' during full fruit ripeness. Cv. 'Rondo' grown under the polytunnel had decreased TPC in leaves. In conclusion, cultivar selection, viticultural practice and leaf-removal time contribute significantly to the accumulation of total polyphenols and resveratrol. Results of this study will contribute to better utilization of biomass produced in the vineyards, help to decrease the negative environmental impacts, and provide an overview on various factors affecting the biochemical constituents, especially in leaves.

Keywords: *Vitis* interspecific hybrids; grapevine leaves; by-products of grape; polyphenols

1. Introduction

Globally, grapevine leaves occur as a major by-product of viticulture practices. These derive from the leaf-removal around the clusters at the beginning of grape colour change (veraison; in grapevine phenological scale, stage 85) [1]. Removing grapevine leaves will help to obtain a high-quality yield, but the leaves are generally recognized to be a major waste in majority of the growing regions [2]. In addition, during pruning of the vines, shoots, leaves and petioles are cut and discarded [3,4]. Therefore, an enormous amount of biomass is produced during winter and summer pruning that remains underutilized, and which is either burnt or composted on the field [5]. Grapevine leaves synthesize a wide range of bioactive secondary metabolites, including polyphenols and stilbenes, which both are important components to ensure key development and growth of the plant even under adverse environmental stress conditions, disease and pest performance, etc. [4,6–10]. The polyphenol compounds have considerable nutraceutical value to be processed and utilized as a functional food or livestock feed ingredient [3,11,12]. Hence, the insignificant plant parts, which are generally discarded, can be explored for valuable polyphenols and other bioactive compounds [13].

Antioxidant properties of grapevine leaves are affected by several factors such as the cultivation region, climatic conditions, viticultural practices, vintage and vine growth stage [2,9,10,13–15]. Moreover, Pantelić et al. [11] have suggested that variations in the chemical composition is a convenient way to differentiate grape leaves of diverse varietal origin. Furthermore, the content of resveratrol (a major polyphenol in grapes) largely varies with genetic background following the certain stilbene metabolic pathway [6,8,16]. The highest content of resveratrol in grapevine leaves was reported to occur during summer, while in stems the increase was noticed during early fall [13]. Even though, many research works have been undertaken in *Vitis vinifera* L. [2,3,10,11,15], there is a lack of scientific knowledge on interspecific hybrids of *Vitis* species in the cooler climate zones. Kedrina-Okutan et al. [14] reported *V. amurensis* to be clearly differentiable with its high concentration of polyphenols throughout the season in comparison to many other *Vitis* sp. (e.g., *V. riparia*, *V. rupestris*, *V. vinifera sylvestris*, etc.). Yin et al. [8] observed that berries of *V. amurensis* emerged from seven different grape accessions regarding their resveratrol content. In certain instances, the results have been contradictory regarding the accumulation of bioactive compounds in different *Vitis* species. For example, the cultivated European type cultivars and their hybrids with *V. labrusca* have shown relatively low contents of total resveratrol [16]. On the contrary, Bábíková et al. [17] found that leaves infected by fungal diseases of blanc interspecific grapevine cultivars to contain more trans-resveratrol than those of noir cultivars compared to healthy leaves. This provides a promising perspective for conducting research on interspecific hybrids of *Vitis* species. The second driver for undertaking this research comes from increased interest from grapevine producers to find practical value addition (valorization) for the discarded biomass.

In this background, the main aim to undertake the study was to find out the effects of (1) different grapevine cultivars ('Rondo', 'Regent', 'Solaris' and 'Boskoop's Glory'), (2) two leaf-removal times (at the beginning of veraison and before fruit harvest; 'Hasansky Sladky' and 'Zilga') and (3) viticultural practice (high polyethylene tunnel and open field; 'Rondo') on the bioactive polyphenols and resveratrol. These results are envisaged to be an immense help for better utilization of valuable biomass produced in the vineyards, to decrease the negative environmental impact of grapevine cultivation and to provide an overview on various factors affecting the biochemical constituents, especially in leaves.

2. Materials and Methods

2.1. Experimental Sites and Plant Material

The experiment was set up under open field conditions at the Experimental Station of the Estonian University of Life Sciences and in Tartu County between 2017 and 2018 under polytunnel and open field conditions. The open field vineyard (58°21′27′′ N, 26°31′16′′ E) was established in 2007 using own-rooted plants of two hybrid grapevine cultivars: 'Hasansky Sladky' (noir; *V. amurensis* × *V. labrusca* × *V. riparia* × *V. vinifera*) and 'Zilga' (noir; *V. amurensis* × *V. labrusca* × *V. vinifera*) and one European grapevine cultivar 'Rondo' (noir; *V. vinifera* L. subsp. *vinifera*). The vines were planted in 2 m × 2 m spaces, trained in low double trunk trellis and 12 buds per plant was left. With cultivar 'Rondo' from 2017 to 2018, there were two variants: (a) field and (b) polytunnel cultivating systems in two locations. The experimental design was a randomised block with 4 replicates and 8 vines in each.

The polytunnel (58° 17′ 1′′ N, 26° 33′ 41′′ E) was established in 2013 using a spacing of 28 m × 7.6 m × 4.6 m (l × w × h) tunnel covered with 0.18 mm thick UV-stable low-density polyethylene. Cultivars in the tunnel experiment were European grapevine cultivars 'Rondo' (noir), 'Regent' (noir; *V. vinifera* L. subsp. *vinifera*), 'Solaris' (blanc; *V. vinifera* L. subsp. *vinifera*) and an interspecific hybrid 'Boskoop's Glory' (noir; *V. vinifera* L. subsp. *Vinifera* × *Vitis* interspecific crossing). The vines were planted in 1.6 m × 2 m spaces, trained in low double trunk trellis and 12 buds per plant was left. White polypropylene fabric was used as a winter cover for the vines. The distance between the experimental areas was approximately 8.5 km.

In both sites, the vine rows were North-to-South oriented, woven ground cover fabric was used in rows, and no additional irrigation system was used neither in tunnel nor in open field conditions. The tunnel air temperature was recorded 24/7 with data loggers, measuring the point at every full hour. Monthly mean temperatures and precipitation are presented in Table 1.

Table 1. Monthly mean temperatures (°C) and precipitation (mm) in the experimental years and in the long-term mean (1981–2010), Tartu-Tõravere station [1] and in polytunnel conditions, Estonia.

	Mean Temperatures, °C					**Precipitation, mm**		
	Open Field			**Polytunnel**		**Open Field**		
Month(s)/Year(s)	**2017**	**2018**	**1981–2010**	**2017**	**2018**	**2017**	**2018**	**1981–2010**
April	3.4	7.2	5.3	4.6	10.2	68	43	36
May	10.2	15.2	11.3	16.1	20.1	28	10	48
June	13.8	15.5	14.9	17.1	20.4	65	66	87
July	15.7	20.2	17.5	20.6	23.6	57	23	83
August	16.5	18.5	16.1	18.8	20.8	112	80	91
September	12.1	14.0	11.0	12.7	15.6	119	99	68
October	5.2	7.2	6.0	6.0	8.6	86	78	81

Note: [1] The weather data according to the web database of Estonian Weather Service (2020).

The leaves were collected two times in both experimental years at the two different phenological stages of grapevines: (a) time of berry softening (stage 85) from the basal part of fruit-bearing canes of the east side of the canopy and (b) when berries were ripe for harvest (stage 89) from the west side of the fruit-bearing canes. Ten to twenty pests-disease-free healthy leaves were collected in three replications. Open field leaves were collected at stages 85 and 89, and polytunnel leaves at stage 89. The identification scale of grape phenological growth stages was used [1]. Fresh leaf samples were frozen immediately (−20 °C) and stored until further analyses.

The soil in both of the experimental areas was high-productivity sandy loam Haplic Luvisol. Soils were sufficiently drained and soil fertility was 45–50 points in the 100-point scale. The field soil nutrient content was determined: P and Mg—excessive, K—high, Ca—medium and pHKCl was 5.4. P, K, Ca and Mg values in the tunnel were high and pHKCl was 5.4. No additional fertilizers were used in either experimental areas.

2.2. Analyses of Total Phenolic Content (TPC) and Resveratrol

TPC was determined by using the method reported by Lambert et al. [18] with slight modifications. Briefly, each of the individual samples (10–20 leaves) was crushed using a 1.5 L glass blender (Stollar Kinetix, Latvia) from which 1 g of homogenous material was weighed into 20 mL tube. Then 10 mL of 50% EtOH +1% HCl was added and the mixture processed for 3 min at maximum speed using the IKA Ultra-Turrax® Tube Drive (IKA®-Werke GmbH & Co. KG, Staufen, Germany). After that the sample was extracted in an ultrasonic bath for 30 min (Branson 1800, Emerson, St. Louis, MO, USA), shaken using a rotator (Multi RS-60, Biosan Sia, Riga, Latvia) and centrifuged at 1000 rpm for 5 min (refrigerated centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). Aliquots were pipetted into the vials for chromatographic analysis and the quantification of total polyphenols, including resveratrol was performed on UHPLC-DAD ACE Excel 3 C18-PFP 100 mm × 2.1 mm (Shimadzu Nexera X2, Kyoto, Japan). The peaks were detected at 280 nm, and the TPC was expressed in mg of chlorogenic acid (CHL) equivalent per 100 g fresh weight (fw) and resveratrol was quantified according to UV-spectra. TPC and individual polyphenols were analysed at the analytical unit of Polli Horticultural Research Centre of Estonian University of Life Sciences (Polli, Estonia).

2.3. Statistical Analysis

The results obtained were tested by a one- and two-way ANOVA. In order to evaluate the effect of the cultivar, grapevine leaf removal time and cultivation site, the least significant differences were calculated by using Fisher's Least Significant Difference (LSD) test, and the different alphabetic letters (a, b . . .) in tables and figure mark significant differences at $p \leq 0.05$. In order to evaluate the mean effect of variables, the results of the two-way ANOVA are presented as a significance level of * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.

3. Results and Discussion

3.1. Effect of Cultivation under Polytunnel Conditions on TPC and Resveratrol

The TPC of leaves collected at full berry maturity varied from 1139 to 1944 mg 100 g^{-1} (fw) in 2017 and from 328 to 553 mg 100 g^{-1} (fw) in 2018 (Table 2). The differences among the tested leaves due to cultivar properties were significant only for 'Regent' in 2017 and 'Solaris' in 2018. The leaves of the noir-fruited 'Regent' showed the highest TPC (1944 mg 100 g^{-1} fw), which was 71% higher when compared to the leaves of blanc-fruited 'Solaris' (the difference was 805 mg 100 g^{-1} fw) in 2017.

Table 2. Total polyphenols and resveratrol in grapevine leaves collected at the stage of harvest (ripe berries) under polytunnel conditions (in 2017–2018).

Cultivar	2017		2018	
	TPC, mg 100 g^{-1} fw	**Resveratrol, mg 100 g^{-1} fw**	**TPC, mg 100 g^{-1} fw**	**Resveratrol, mg 100 g^{-1} fw**
'Rondo'	1159 ^{b}	0.095 ^{b}	342 ^{b}	0.216 ^{a}
'Regent'	1944 ^{a}	0.272 ^{a}	427 ^{b}	0.235 ^{a}
'Boskoop's Glory'	1237 ^{b}	0.063 ^{b}	328 ^{b}	0.282 ^{a}
'Solaris'	1139 ^{b}	0.116 ^{b}	553 ^{a}	0.178 ^{a}
	TPC, mg 100 g^{-1} fw	**Resveratrol, mg 100 g^{-1} fw**		
Mean effect of year	***	*		
Mean effect of cultivar	***	*		

Note: TPC—total phenolic content, mg 100 g^{-1} in fresh weight (fw); different letters (a, b) in columns mark significant differences at $p \leq 0.05$. Results of the two-way-ANOVA: significance for the mean effect of the cultivation year and cultivar, * $p \leq 0.05$ and *** $p \leq 0.001$.

Fernandes et al. [19] reported similar findings and detected an increased level of polyphenols in the leaves of grapevines with noir fruits when compared to the leaves of blanc ones. In the present experiment, the leaves of blanc 'Solaris' showed an increased leaf TPC (in 2018) when compared to the rest, noir ones. In addition, blanc grapevine cultivars have been demonstrated to contain the high amounts of polyphenols detected in stems and leaves than noir ones [2,13]. In the present experiment, variations in the TPC compared between two experimental years were significant. The mean effect of the year was significant for leaf TPC at $p \leq 0.001$ and for resveratrol at $p \leq 0.05$. Maante-Kuljus et al. [20] obtained comparable results with grape berries, which showed enormous yearly fluctuations in the TPC. One of the possible reasons could be related to high temperatures from April to October in 2018 in polytunnel conditions when compared to the year 2017 (see Table 1). The sensitivity of different cultivars to temperature fluctuations affecting the accumulation of polyphenols has been demonstrated earlier [21], especially the decreasing effect at an elevated temperature range [22].

The content of resveratrol in polytunnel conditions ranged between 0.06 and 0.27 mg 100 g^{-1} (fw) in 2017 and from 0.18 to 0.28 mg 100 g^{-1} (fw) in 2018 (Table 2). 'Regent' cv. had the highest content of resveratrol (27 mg 100 g^{-1} fw) in the leaves in 2017, but there were no significant differences among the grape cultivars in 2018. The mean effect of the cultivar was significant for grapevine leaf TPC at $p \leq 0.001$ and for resveratrol at $p \leq 0.05$. The contents of the total resveratrol have been declared to be much lower in leaves than in fruit skins, but still the amounts are considerable [16]. Wojdyło et al. [23] have presented significant effects of cultivar properties of interspecific hybrid grapevines on their biochemical composition. Resveratrol composition is largely dependent on the genetic background of the cultivars [16]. Also, high peak of resveratrol in grapevine leaves occurred mainly during the berry ripening stage [13]. According to Doshi et al. [3], total polyphenols in *V. vinifera* L. are maximum at the initial stage of berry development as well as in berry stems, leaves, petioles and shoots, indicating the protective roles of these vine parts during early stages of berry development. This signifies the increased polyphenols and resveratrol in certain cultivars in relation to the initial physiological status of the grapevine and its genetic predisposition to synthesize these compounds.

3.2. Effect of Harvest Time under Open Field Conditions

The results obtained in this study showed that TPC and resveratrol contents were dependent on the leaf-harvest time (Table 3). In both cultivars tested, the tendency for increased TPC and resveratrol was evident for the second leaf-harvest time (stage 89). Autumn-collected leaves had a significantly higher amount of polyphenols and resveratrol when compared to summer leaves (stage 85). The effect was significant for both parameters in cv. 'Zilga' during both experimental years, but for TPC observed in 'Hasansky Sladky' cv. only in 2018, and resveratrol in both years. The mean effect of the leaf harvest time was significant in both experimental years for both parameters analysed.

Table 3. Total polyphenols and resveratrol in grapevine leaves according to the cultivar and leaf-harvest time in open field conditions in 2017–2018.

Leaf-Harvest Time	**Cultivar**	**2017**		**2018**	
		TPC, mg 100 g^{-1} fw	**Resveratrol, mg 100 g^{-1} fw**	**TPC, mg 100 g^{-1} fw**	**Resveratrol, mg 100 g^{-1} fw**
Stage 85	'Zilga'	1264 ^{b}	0.167 ^{b}	351 ^{c}	0.206 ^{c}
Stage 89	'Zilga'	1841 ^{a}	1.061 ^{a}	464 ^{a}	1.971 ^{a}
Stage 85	'Hasansky Sladky'	1163 ^{b}	0.222 ^{b}	395 ^{abc}	0.274 ^{c}
Stage 89	'Hasansky Sladky'	1213 ^{b}	0.882 ^{a}	444 ^{ab}	0.678 ^{b}
Mean effect of leaf harvest time		***	**	*	***
Mean effect of cultivar		***	-	-	***

Note: Phenological stages of grapevine, stage 85—berry softening, stage 89—ripe berries (according to [1]). TPC—total phenolic content, mg 100 g^{-1} in fresh weight (fw); different letters (a, b, c) in columns mark significant differences at $p \leq 0.05$. Results of a two-way-ANOVA: significance for mean effect of harvest time and cultivar, * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.

In the phenological scale, during the grapevine developmental stage 89 [1], the content of resveratrol in cv. 'Zilga' increased up to 0.98 mg in 2017 and up to 1.76 mg in 2018 when compared to summer leaves. The variability of resveratrol in 'Hasansky Sladky' cv. among leaf harvesting times was also significant, but with lower yearly fluctuations in contents. Eftekhari et al. [13] found that the harvest time is an essential factor that can have influence on the polyphenolic composition in vine shoots and leaves. Martín-Tornero et al. [15] confirmed the patterns of polyphenols in grapevine leaves to show significant differences during the grape maturation period between the months of June and September. In addition, the leaves were in the mature phase, which meant the peak of photosynthesis was to be at its highest in fully open leaves up to 40 days [24]. Polyphenols as plant secondary metabolites are not essential for the plant survival, but are synthesised more or less during a plants life cycle for different reasons, for example due to abiotic stress conditions [6–8,25]. Autumn leaves (in mature phase) have a stress-induced increase in polyphenolic compounds due to cooler night temperatures in open field conditions. Mean temperatures in October were below +10 °C (Table 1), which favoured the leaf colouring and affected the contents.

3.3. *Effect of the Cultivation Site on the Content of Polyphenols and Resveratrol in 'Rondo'*

Cultivation site significantly affected the content of polyphenols in 'Rondo' cv. grapevine leaves in both experimental years (Figure 1; *** $p \leq 0.001$). TPC of open field vine leaves (1094 mg 100 g^{-1}) was 31% higher when compared to polytunnel ones (750 mg 100 g^{-1}). Leaves of cv. 'Rondo' had an opposite effect when compared to fruits—open field leaves had increased polyphenols, because stress factors are more evident in open field conditions. In open field and in polytunnel conditions the abiotic factors such as light, temperature, humidity, wind, CO_2, soil conditions, etc., are of high importance, which can affect the plants. The vegetation period of vines tend to be longer in soils with high humus content, which shift the fruit ripening and prolong the lifespan of the leaves as well. In our experiment, the soils of both experimental sites had a high-productivity score.

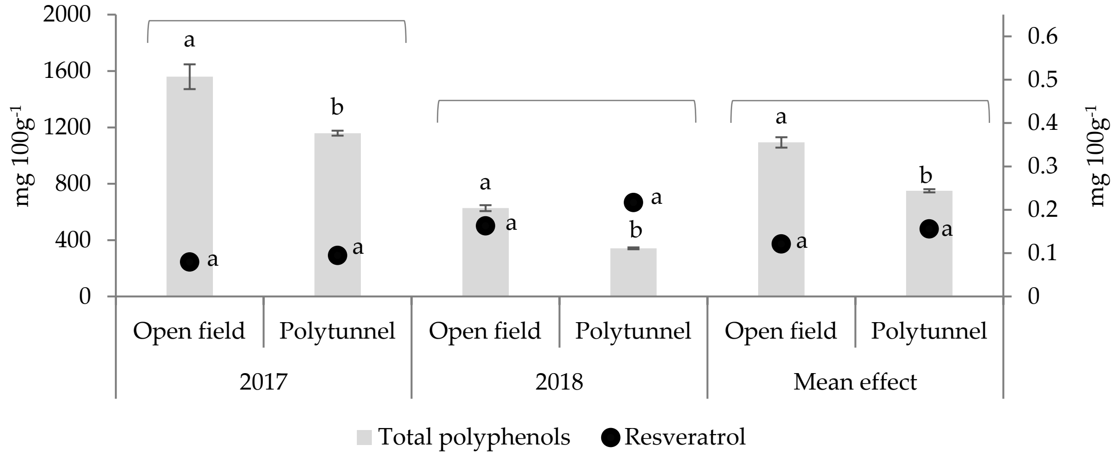

Figure 1. Total polyphenols (in columns; mg 100 g^{-1} chlorogenic acid eqv.) and resveratrol (with dots; mg 100 g^{-1} cyanidin-3-glucoside eqv.) according to the viticultural practice (open field and polytunnel) in the leaves of the grapevine cultivar 'Rondo' in 2017–2018 and the two years mean. Different letters on columns (a, b) mark significant differences, at $p \leq 0.05$. Results of the two-way-ANOVA: significance of the mean effect of viticultural practise, at $p \leq 0.001$.

Resveratrol in vine leaves varied from 0.080 to 0.095 mg 100 g^{-1} (fw) in 2017, and from 0.163 to 0.216 mg 100 g^{-1} (fw) in 2018. However, viticultural practice did not have any significant effect on vine leaf resveratrol, though the tendencies to have higher contents revealed in polytunnel conditions. In earlier experiments, the effect was evident in the fruits as well. As shown in Figure 1, an increase in resveratrol during the year 2018 can be attributed to a year with elevated temperatures (from 20.1 to 23.6 °C), when compared to open field temperatures (from 15.2 to 20.2 °C). Moreover, the polytunnel is a closed system in which the temperatures are significantly higher compared to the open field [9], and this plays an important role in increasing the contents. In addition, the diffuse plastic induce greater variations in leaf area and leaf chlorophyll content as compared to uncovered vines [9]. The vines' exposure to light can enhance polyphenols accumulation in leaf tissues [2,7,8]. Besides, the radiation received by leaves can change the phenolic content too [2,6,26]. Yin et al. [8] claimed the resveratrol metabolic pathways to be driven by ultraviolet-C radiation, which an increase occurs with elevated temperatures and exposure to sunlight. In the present experiment, the mean effect of the viticultural practice on the total polyphenols and resveratrol was significant at $p \leq 0.001$. Therefore, the differences in cultivar properties and their behaviour in variable conditions while selecting viticultural practice and suitable cultivars needs to be carefully considered [20]. The grapevine cultivar 'Rondo' revealed its leaf total polyphenol potential in open field conditions, presenting the increased contents in both experimental years when compared to polytunnel conditions.

4. Conclusions

Grapevine cultivation and viticulture practices produce an enormous amount of biomass during winter and summer pruning. This kind of waste (biomass) is generated in high volumes but usually burnt or composted on-field. In addition to the canes and shoots, the grapevine leaves constitute the majority of the residues as the process of leaf-removal starts at the beginning of veraison. The biochemical composition of leaves vary significantly even in cold resistant hybrid grapevine cultivars and show higher contents, which depends on the cultivar in open field or polytunnel conditions.

In the present experiment, the effect of cultivar varied annually, cv. 'Regent' had increased total polyphenols and resveratrol in 2017, but 'Solaris' in 2018. Leaves collected during full fruit ripeness in open field conditions cvs. 'Zilga' and 'Hasansky Sladky' presented higher content of total phenolics and resveratrol. Autumn leaves in the mature stage produced more polyphenolic compounds during cool night temperatures and shorter photoperiod conditions. Viticultural practices (polytunnel) decreased the polyphenols in leaves. Leaf resveratrol presented greater variability rather in open field conditions. In conclusion, cultivar selection, viticultural practice and leaf-removal time contribute significantly to the accumulation of total polyphenols and resveratrol. The current study revealed high potential of the leaves of interspecific hybrid grapevine cultivars, to hold high promise to be used for value addition and can find practical applications for developing novel food or livestock feed based products or for cosmetic and/or pharmaceutical applications. The data generated in this study is envisaged to be continued further focusing mainly on improving the extraction procedures, especially use of green extraction techniques. Further research is also warranted based on observations of this study, which is envisioned to efficiently support concepts of zero waste and circular economy in EU.

Author Contributions: Conceptualization, methodology, K.K. and R.R.; formal analysis, R.R., M.M.-K. and A.A.; data curation, R.R. and K.K.; writing—original draft preparation, R.R. and K.K.; writing—review and editing, R.B.; visualization, R.R.; supervision, K.K. and R.B.; project administration and funding acquisition, K.K. and R.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 810630 (Valortech). This research was also supported by the "Innovation Cluster" of the Estonian Rural Development Plan (ERDP Action 16 'Cooperation') 2014–2020, financially supported by European Agricultural Fund for Rural Development.

Acknowledgments: European Regional Development Fund is acknowledged for supporting collaboration.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript or in the decision to publish the results.

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