Italian Journal of Animal Science

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tjas20

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To cite this article: Davide Lanzoni, Francesca Mercogliano, Raffaella Rebucci, Corinne Bani, Luciano Pinotti, Chiara Di Lorenzo, Giovanni Savoini, Antonella Baldi & Carlotta Giromini (2024) Phenolic profile and antioxidant activity of hemp co-products following green chemical extraction and ex vivo digestion, Italian Journal of Animal Science, 23:1, 651-663, DOI: 10.1080/1828051X.2024.2348098

To link to this article: https://doi.org/10.1080/1828051X.2024.2348098

Published online: 14 May 2024.

ITALIAN JOURNAL OF ANIMAL SCIENCE
2024, VOL. 23, NO. 1, 651–663
https://doi.org/10.1080/1828051X.2024.2348098

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RESEARCH ARTICLE

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Phenolic profile and antioxidant activity of hemp co-products following green chemical extraction and ex vivo digestion

Davide Lanzoni, Francesca Mercogliano[b], Raffaella Rebucci, Corinne Bani[b], Luciano Pinotti[a,c], Chiara Di Lorenzo[b,c], Giovanni Savoini, Antonella Baldi and Carlotta Giromini[c]

[a]Dipartimento di Medicina Veterinaria e Scienze Animali (DIVAS), Università degli Studi di Milano, Lodi, Italia; [b]Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milano, Italia; [c]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, Milano, Italy

ABSTRACT

The valorisation of biomass generated along the agri-food chain into co-products represents a valid approach to produce alternative and sustainable feed ingredients. Although to date, some co-products have already been included in the diets of livestock animals, the characterisation of hemp co-products (Cannabis sativa L.) is still at an early stage. For this, the aim of this this work was to investigate the nutritional and functional profile [total phenolic content (TPC) and antioxidant activity (ABTS, FRAP)] of hemp co-products [mix flowers and leaves (MFL), hulls (HLs) and hempseeds cake], after green chemical extraction (0% EtOH; 50% EtOH; 100% EtOH) and *ex vivo* digestion process. High-Performance Thin Layer Chromatography was performed on 50% EtOH chemical extracts to identify the main phenolic compounds. The results reported an interesting nutritional profile, although dependent on the processing to obtain the co-product. The functional aspect of the chemical extracts showed a higher TPC for MFL, especially after 50% EtOH extraction (3551.12 ± 98.54 mg TAE/100 g) identifying Quercetin, Kaempferol, Rutin, Chlorogenic acid, Ferulic acid, Cannabigerol and α-tocopherol as the main compounds present, a profile similar to that of HLs. This was also observed for ABTS (6950.10 ± 546.82 mg TE/100 g) and FRAP (130.05 ± 4.67 mg FeSO4/100 g). *Ex vivo* digestion confirmed the high functional profile of the two matrices. Despite this, it is important to emphasise that processing co-products are characterised by a high variability that may lead to different results and effects on the health and performance of monogastric animals.

HIGHLIGHTS

- The high environmental impact of the food/feed sector has prompted scientific research to valorise the co-products of the food chain.
- Hemp co-products are characterised by an interesting nutritional profile, particularly in terms of protein and fat content.
- Hemp co-products, particularly hulls and mix flowers/leaves are characterised by high functional activity.

ARTICLE HISTORY
Received 28 January 2024
Revised 27 March 2024
Accepted 22 April 2024

KEYWORDS
Antioxidant; extraction; digestion; hemp; phenols

Introduction

The high environmental impact of the food and especially feed sector is a highly debated topic in recent years, which is attracting increasing interest from the scientific community, particularly from the perspective of food-feed competition. Globally, the production, processing, and transport of feed for the livestock sector accounts for 45% of the total production of greenhouse gases (Makkar, 2018). Simultaneously, as reported by the same author, the area dedicated to feed-crop cultivation accounts for 33% of the total arable land, while about 30% is occupied by grazing, with more than 90% water consumption (Makkar, 2018). This scenario is bound to worsen if considering that the world's current population will exceed 9 billion people in the next 30 years, leading to an ever-increasing need for food and food production, which could have a further impact on climate change, as shown by projections for 2050 by the EAT-Lance commission (Willet et al. 2019). For this reason, the

CONTACT Davide Lanzoni davide.lanzoni@unimi.it

European Union (EU) is working to ensure a sustainable future through the implementation of strategies such as the Green Deal and One Health, the aim of which is the transformation of the EU into a prosperous society with a competitive, clean, and circular economy that recognises the close link between human, animal, and environmental health (Sinclair, 2019; Fetting, 2020; Pinotti et al., 2023). In this context, the Farm to Fork (F2F) strategy, the heart of the European Green Deal, plays a major role. F2F is a 10-year plan devised by the European Commission to guide the agricultural transition towards more equitable, healthy, and environmentally friendly food systems, making them more sustainable than they are today (European Union, 2020). To achieve these goals, one possible approach is to utilise the huge amount of non-edible biomass produced along the entire food chain (about 1.3 billion tonnes) as co-products for livestock (FAO, 2011; Rakita et al., 2021). As reported by Pinotti et al. (2020), the term co-product refers to any product obtained from different agro-industrial processes. To date, several co-products such as beet pulp, soybean meal, soybean molasses, sunflower meal, and grape marcs (GMs) have already been included in animal diets due to their interesting nutritional profile, but many others could be used (Mirzaei-Aghsaghali and Maheri-Sis, 2008; Vastolo et al., 2022). Among these, the co-products of industrial hemp (*Cannabis sativa* L.) are attracting increasing interest. Hemp is a dicotyledonous, annual, herbaceous, angiosperm plant that is widespread worldwide due to its easy adaptability (Lanzoni et al., 2023a, 2023b). It is considered a low environmental impact plant as it does not require large amounts of water, use of pesticides and acts as an antagonist to weeds (Rupasinghe et al., 2020). At the same time, as well as being able to grow rapidly in different agro-ecological conditions, it is an excellent candidate for carbon sequestration due to its height (it can reach up to four metres) (Farinon et al., 2020; Rupasinghe et al., 2020; Rehman et al., 2021).

Following EU Regulation No. 1307/2013, which allows the cultivation of hempseeds (HSs), on condition that they are registered in the European catalogue and have a Δ9-tetrahydrocannabinol (THC) content of less than 0.2%, this plant has been attracting the interest of several industries (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2011; Lanzoni et al., 2023a). In particular, the food and feed industry has studied the properties of HS, previously considered a waste product from the processing of this plant, recognising it as a feed material with important nutritional and functional properties (Farinon et al., 2020; Lanzoni et al., 2023a). In the F2F contest, hemp shows as a valuable opportunity to valorise the co-products of its processing. As reported by Ely and Fike (2022), Vastolo et al. (2021), and Lanzoni et al. (2023b), the hemp co-products can be summarised as follows: whole plant, stems, chaffs, stalks, hulls (HLs), HS cake (HSC), leaves (before and after cannabinoid extraction), and flowers (before and after cannabinoid extraction). As reported by the same authors, these co-products are characterised by an interesting nutritional profile, particularly regarding to protein content, which starts at 5% for stalks and reaches values of more than 20% for HSC, chaffs, flowers, and HLs. Interesting results are reported also for the lipid content (Vastolo et al., 2021; Ely and Fike, 2022). However, their nutritional and especially functional profile is still poorly characterised.

This is probably due to the fact that hemp cultivation is still small-scale, even though Europe, as reported by Horne (2020), is one of the main countries in terms of hemp production area. Furthermore, as reported by Ely and Fike (2022), this is because the various hemp industries are in their early stages, without significant markets capable of absorbing large quantities of hemp scraps and consequently generating large volumes of co-products. To date, the EU, through Regulation 2022/1104 of 1 July 2022, has registered only HS, HSC, HS oil, hemp flour and hemp fibre (both originating from stems) in the European Catalogue of feed materials (European Union (EU)), 2022).

On this basis, the aim of this work was to investigate the nutritional and functional profile of hemp co-products (mix flowers/leaves (MFL), HLs and HSC), also in comparison to GMs, matrices highly described in the literature, to assess their potential for livestock feed. In particular, total phenolic content (TPC) and antioxidant activity [2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS); Ferric Reducing Antioxidant Power (FRAP); 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH)] were monitored following a green chemical extraction, also identifying the main phenolic compounds by High-Performance Thin-Layer Chromatography (HPTLC). In parallel, TPC, ABTS, and FRAP were also evaluated following an *ex vivo* digestion protocol.

Material and Methods

Material

- Hulls: HLs are derived from the dehulling process of HSs using sieves and airflow (*C. sativa* L, variety

Bialobrzeskie). The HSs were supplied by a Czech company (Chrastice, Czech Republic). Specifically, the HSs were sown in May 2022 and harvested in early October 2022.

- Mix flowers/leaves: The MFL (*C. sativa* L, variety Carmagnola) was provided by a local company (CN, Italy). After hand-harvesting, the sample was dried slowly in the dark in a closed and ventilated environment in order to safeguard the functional compounds. Subsequently, the cannabidiol (CBD) content was recorded and resulted in a concentration of between 2-3%.
- HSs cake: The HSC (*C. sativa* L, variety Carmagnola) was supplied by a local company (CN, Italy). Specifically, the seeds were sown in May 2022 and harvested in October 2022 by threshing. Following the harvesting process, the seeds were dried to stabilise the moisture content. Finally, they were cold-pressed for the production of HSs oil. The remaining fraction obtained (HSC) was harvested and stored in a dry and ventilated environment.
- Grape marcs: The GMs (from red wine industry, also containing seeds) were provided by a local producer (PD, Italy).

Methods

Chemical analysis

Chemical analysis was performed following the Official Methods of Analysis (AOAC, 2005). In particular, dry matter (DM) and ashes were determined according to AOAC method 942.05. Protein content and ether extract were assessed with the AOAC method 2001.11 and DM 21/12/1998, respectively. Finally, the fibrous fractions were identified according to Van Soest et al. (1991).

Green chemical extractions

Green chemical extractions were performed using three different ratios of water:ethanol (H_2O:EtOH) (0% EtOH; 50% EtOH; 100% EtOH). More precisely, the protocol developed by Brighenti et al. (2017) was performed with minor modifications (Lanzoni et al., 2024). Following grinding (diameter of 1 mm, rotor mill Retsch Mod. zm 200, Hann, Germany) of the samples (0.150 ± 0.05 g), 5 mL of solvent (H_2O:EtOH) were added and incubated for 1 h at room temperature (RT), under shaking conditions in the dark. At the end of the incubation, the samples were centrifuged at 4000 rpm for 5 min obtaining two fractions: (I) supernatant was recovered and stored at 4 °C, (II) precipitate, corresponding to the residual fraction, was extracted twice, obtaining a final volume of extract equal to 15 mL per sample. The final extract obtained was stored at −20 °C until further analysis (TPC and antioxidant activity). Each extraction was performed in triplicate ($n = 3$). For each biological replicate, the technical duplicate was considered.

Total phenolic content and antioxidant activity (ABTS and FRAP assays)

The protocol of Attard (2013) with minor modifications (Lanzoni et al., 2023a) was used to test TPC. Specifically, 200 µL of each sample were incubated with 1.0 mL of Folin-Ciocalteu reagent and 800 µL of sodium carbonate in the dark at RT for 20 min. At the end of the incubation, the samples were read with a spectrophotometer at a wavelength of 630 nm. Values were expressed in terms of Tannic acid equivalent (mg TAE/100 g of dried material).

At the same time, antioxidant activity was assessed using the ABTS (Re et al., 1999) and FRAP (Abdelaleem and Elbassiony, 2021) methods. For ABTS, 10 µL of sample were added to 1.0 mL of diluted $ABTS^{\circ+}$ solution ($A_{734nm} = 0.700 \pm 0.020$), incubated for 6 min at RT in the dark, and read at 734 nm. Data were expressed as Trolox equivalent (mg TE/100 g of dried material).

For FRAP, the working solution (FRAP reagent) was prepared as follows: a) 25 mL of acetate buffer (300 mM; pH 3.6); b) 2.5 mL of 2,4,6-tripyridyl-s- triazine (10 mM); c) 2.5 mL of $FeCl_3$ (20 mM). Next, 10 µL of each sample were added to 300 µL of FRAP reagent and incubated at RT for 10 min in the dark and read at 595 nm. Results were expressed as mg $FeSO_4$/100 g of dried material.

High-performance thin layer chromatography and dpph of green chemical extractions

In this study, the HPTLC technique was used to analyse flavonoids, phenolic acids, Cannabigerol (CBG) and α-tocopherol after chemical extraction with 50% EtOH, as a high TPC was obtained with this ratio. Gallic acid, Chlorogenic acid, Caffeic acid, Ferulic acid, Ruthin, Epicatechin, and α-tocopherol standards were purchased from Sigma Aldrich (Steinheim, Germany), while Quercetin 3-O-glucoside, Kaempferol, and Catechin were purchased from Extrasynthese (Genay, France). Cannabigerol by Linnea Natural Pharma Solutions (Riazzino, Switzerland).

In particular, following the extraction process, 5 mL of each sample were centrifuged at 3000 rcf at 4 °C for 10 min (5810 R, Eppendorf, Hamburg, Germany). After centrifugation, the samples were filtered using

0.45 μm PTFE filters (VWE) and dried with nitrogen flow to concentrate the compounds and solubilise them in 200 μL of methanol (VWR International, Fontenay-sous-Bois, France), resulting in a final concentration of 250 mg/mL. Subsequently, 10 μL of each sample were loaded onto an HPTLC silica-gel plate 60 F254 (10 × 20 cm, Merck, Darmstadt, Germany) with 5 μL of Quercetin 3-O-glucoside, Rutin, Catechin, Epicatechin, Gallic acid, Caffeic acid, Chlorogenic acid, Ferulic acid, CBG and α-tocopherol standard solution, by a semi-automatic sample applicator (Linomat 4, CAMAG, Muttenz, Switzerland). For all standards used, the concentration was 200 μg/mL, with the exception of CBG (1 mg/mL). After the chromatographic run, where the mobile phase consisted of 10 mL of acetone:toluene:formic acid (4.5:4.5:1 v/v/v) (VWR International, Fontenay-sous-Bois, France), the plate was exposed to UV light at 254 and 366 nm.

Subsequently, the plate was derivatised with a 0.05% DPPH methanolic solution (Sigma Aldrich Steinheim, Germany), kept in the dark for 30 min, and then examined under visible light using VisionCats software (CAMAG, Muttenz, Switzerland).

Ex vivo digestion process

The digestion process was adapted from Devle et al. (2014) and performed as reported by Lanzoni et al. (2024). More precisely, gastric and intestinal fluids were collected from pigs ($n = 20$) at slaughter between 50 and 110 days of age. Then, in order to remove the undigested fraction, the fluids were centrifuged for 10 min at 4000 rcf and used to pool gastric and intestinal fluids to reduce variability. The resulting fluids were frozen at −20 °C for up to 48h. Before digestion, pH and enzyme activity were checked and adjusted if necessary to match gastric and intestinal conditions. As reported in Figure 1, at the beginning of the gastric phase (0h), at the end of the gastric phase (2h), and at the end of the intestinal phase (4h), aliquots (1 mL) were taken to measure TPC and antioxidant activity (ABTS and FRAP) during *ex vivo* digestion. The digestion process was replicated three times ($n = 3$) taking two technical replicates for each digestion step.

Statistical analysis

Total phenolic content and antioxidant activities of the green chemical extracts and *ex vivo* digestion were analysed by two-way Anova (*EtOH concentration x Co-products*; *Time x Co-products*, respectively) followed by Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). All data are reported as means ± standard error of the mean (SEM) of at least three independent experiments. Values are considered statistically significant for a 95% confidence interval (*p-value* = 0.05).

Results and discussion

Chemical analysis

The nutritional profile of HLs, MFL, HSC, and GMs is shown in Table 1.

As reported in Table 1, hemp co-products showed an interesting nutritional profile, even when compared to GMs, a co-product of the wine industry, which is highly used in the feed sector. In general, the results obtained in this study are partially confirmed in the literature (Ely and Fike, 2022; Vastolo et al., 2022; Kleinhenz et al., 2020). As reported by Vastolo et al. (2022), these differences are probably due, not only to the genotype of the plant, but also to harvesting, agrological, and environmental conditions. In more detail, MFL showed a high ash content. Kleinhenz et al. (2020) demonstrated that hemp leaves are characterised by an ash content of 21.2% while flowers at

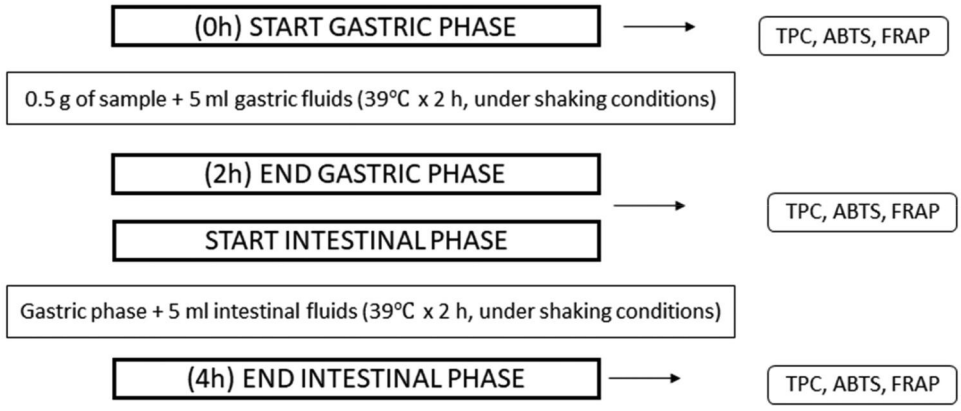

Figure 1. Workflow of *ex vivo* digestion protocol (Devle et al., 2014; Lanzoni et al., 2024). TPC: Total phenolic content.

Table 1. Chemical composition of MFLs (mix flowers/leaves), HLs (hulls), HSC (Hemp seed cake) and GMs (grape marcs).

Sample	DM	ASHES	CP	EE	NDF	ADF	ADL
MFL	94.58 ± 0.43	17.40 ± 0.19	15.79 ± 0.02	12.02 ± 0.37	42.06 ± 0.31	18.05 ± 0.72	5.34 ± 0.15
HLs	91.28 ± 2.11	12.03 ± 0.31	14.49 ± 0.75	9.85 ± 0.04	44.70 ± 1.47	29.30 ± 0.50	10.67 ± 0.11
HSC	94.46 ± 0.05	8.27 ± 0.85	32.54 ± 0.71	7.69 ± 0.05	52.59 ± 1.84	29.00 ± 1.59	12.14 ± 0.95
GMs	94.02 ± 0.02	7.57 ± 0.14	10.69 ± 0.27	8.33 ± 0.62	55.49 ± 0.66	47.54 ± 0.12	35.47 ± 0.47

Data are presented as mean ± standard error of mean (SEM). ($n = 3$). DM : dry matter; CP : crude protein; EE : ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin. Ashes, CP, EE, NDF, ADF and ADL are expressed in % w/w on DM basis.

14.1%, suggesting that a combination of them results in a reduction of the final percentage (intermediate in our case). The high ashes values are due to the presence of minerals such as calcium, phosphorus, magnesium, potassium, and sulphur, which are known to be high in hemp leaves and flowers (Kleinhenz et al., 2020) and which, in turn, may influence the digestibility, as shown by Lanzoni et al. (2023a). As reported in Table 1, HSC showed a high protein content (32.54 ± 0.71%). This feature is related to the treatment adopted for obtaining the specific co-product. In this case, the removal of oil (one of the main components of whole HSs) led to an increase in the total protein content (from 30% to 50%), as demonstrated by House et al. (2010) and Ely and Fike (2022). In parallel, HLs and MFL were characterised by a highly comparable protein content (14.49 ± 0.75%; 15.79 ± 0.02%, respectively), however lower than reported by Ely and Fike (2022). This difference, as previously reported, is due to the variability factors listed by Vastolo et al., (2022). It is important to emphasise that, although HLs are a matrix obtained following the removal of the fibrous outer layer of HSs (dehulling process), they showed a high protein content. As reported by House (2021), this could be due to the fact that, during the dehulling process, part of the HSs endosperm (rich in protein and lipids) is also inevitably removed, thus increasing the final protein content.

This also explains the high lipid content observed in HLs (9.85 ± 0.04%), highly comparable with GMs (8.33 ± 0.62%), confirming the values reported by House et al. (2010) and Baumgärtel et al. (2007). Among the matrices studied, the MFL showed the highest lipid content (12.02 ± 0.37%), confirming the values observed by Ely and Fike (2022). This result could be justified by the presence of essential oil in the hemp flowers, which is highly used in the cosmetic and pharmaceutical sectors for its important functional properties (Farinon et al., 2020).

All analysed matrices presented high fibre content, expressed as Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin (ADL). Highest values for all analysed fractions were obtained for HSC. In particular, NDF measured in HSC was close to GMs, confirming, at least partially, what was observed by Halle and Shöne (2013). More precisely, the authors reported a percentage of NDF, ADF, and ADL of 45%, 30% and 12% (expressed on DM basis) respectively, values comparable with those reported in Table 1. In parallel, among the tested samples, MFL showed the lowest values for NDF (42.06 ± 0.31%), ADF (18.05 ± 0.72%), and ADL (5.34 ± 0.15%). Although there is no data in the literature on the nutritional composition of the MFL, it is evident that the values observed in the present study are a combination of the results obtained from the analysis of the matrices tested individually, as reported by Kleinhenz et al. (2020) and Ely and Fike (2022). Finally, the HLs showed a lower content of NDF, ADF, ADL than observed by House et al. (2010), Kim and Nyachoti (2017), and Kim et al. (2018). More precisely, the authors showed that NDF in these matrices can reach 50–65% and 38–50% of ADF (on DM basis). Again, the observed differences can be attributed to genotype, collection, agrological and environmental differences.

Although high levels of fibre may affect the total digestibility, it is important to emphasise that its adequate consumption may have a functional, especially probiotic, role, as reported by Farinon et al. (2020).

Total phenolic content and antioxidant activity (ABTS and FRAP) of green chemical extracts

Scientific research is increasingly investigating the properties of phenolic compounds due to their associated benefits, which can be exploited in the formulation of functional foods and nutraceuticals (Abdalla et al., 2007). The properties of these compounds can be influenced by the type of extraction. To date, solvent-based extraction is one of the most widely used on a laboratory scale due to its speed and cost-effectiveness. The efficacy of this type of extraction depends mainly on the solvent used, identified by Rotta et al. (2017) as one of the main critical parameters due to the different chemical characteristics and polarity of the phenolic compounds. For this reason, as shown in Figure 2a, we decided to study the TPC of hemp co-

products, using three different ratios of H_2O:EtOH, as previously reported.

As shown, 50% EtOH extraction (3551.12 ± 98.54 mg TAE/100g) resulted in significantly higher TPC ($p < 0.05$) for MFL. This was also observed for HLs (1647.11 ± 60.53 mg TAE/100g) compared to 100% EtOH (600.53 ± 23.55 mg TAE/100g). This trend, although not statistically significant compared to other ratios, was also reported for HSCs and GMs, partially confirming what has been reported in the literature (Prasad et al., 2009; Özbek et al., 2020). More specifically, Prasad et al. (2009) showed that the extraction rate of longan fruit pericarp increased with increasing EtOH concentration from 25% to 50% and decreased for concentrations higher than 75%. A similar result was obtained by Özbek et al. (2020) on pistachio HLs. Most probably, this trend can be explained by the '*like dissolves like principle*', where H_2O allows polar compounds to dissolve, while EtOH, an organic solvent, dissolves less polar ones, resulting in a higher extraction rate (Lim et al., 2019).

Although Özbek et al. (2020) reported a lower extraction rate with 100% EtOH than with 100% H_2O, it is evident in Figure 2a, how this depends on the matrix analysed. Indeed, as reported by Robbins (2003), in addition to the solvent used, sample-solvent ratio, pH, temperature, and extraction time, the nature of the compounds are important factors to consider, as they can influence the extraction rate.

Indeed, considering the individual matrices (Figure 2a), MFL showed significantly higher values ($p < 0.05$) in each concentration tested, except in comparison with HLs for 0% EtOH extraction. Although, the phenolic profile of the leaves is little explored in the literature, that of the flowers has been characterised by Izzo et al. (2020a). The authors reported how hemp flowers can be used as new resources for nutraceutical formulations, thanks to the high presence of phenols, in particular hydroxycinnamic acids (chlorogenic, p-coumaric, ferulic acids) and especially flavonoids. More precisely, Izzo et al (2020a) demonstrated how these compounds are 10 to 100 times more present in flowers than in any other part of hemp, thus explaining the high TPC levels, expressed as mg TAE/100 g, shown in Figure 2a.

At the same time, the results obtained for HLs in each extraction (999.65 ± 13.58 mg TAE/100g; 1647.1 ± 50.53 mg TAE/100g; 600.53 ± 24.55 mg TAE/100g, for 0%, 50% and 100% EtOH, respectively), highly comparable with those of GMs, showed higher values than those obtained in a previous our publication for HSs (550.3 ± 28.27 mg TAE/100g) extracted in pure methanol, a stronger solvent than EtOH (Lanzoni et al., 2023a). This result is most probably due to the fact that phenolic compounds are more concentrated

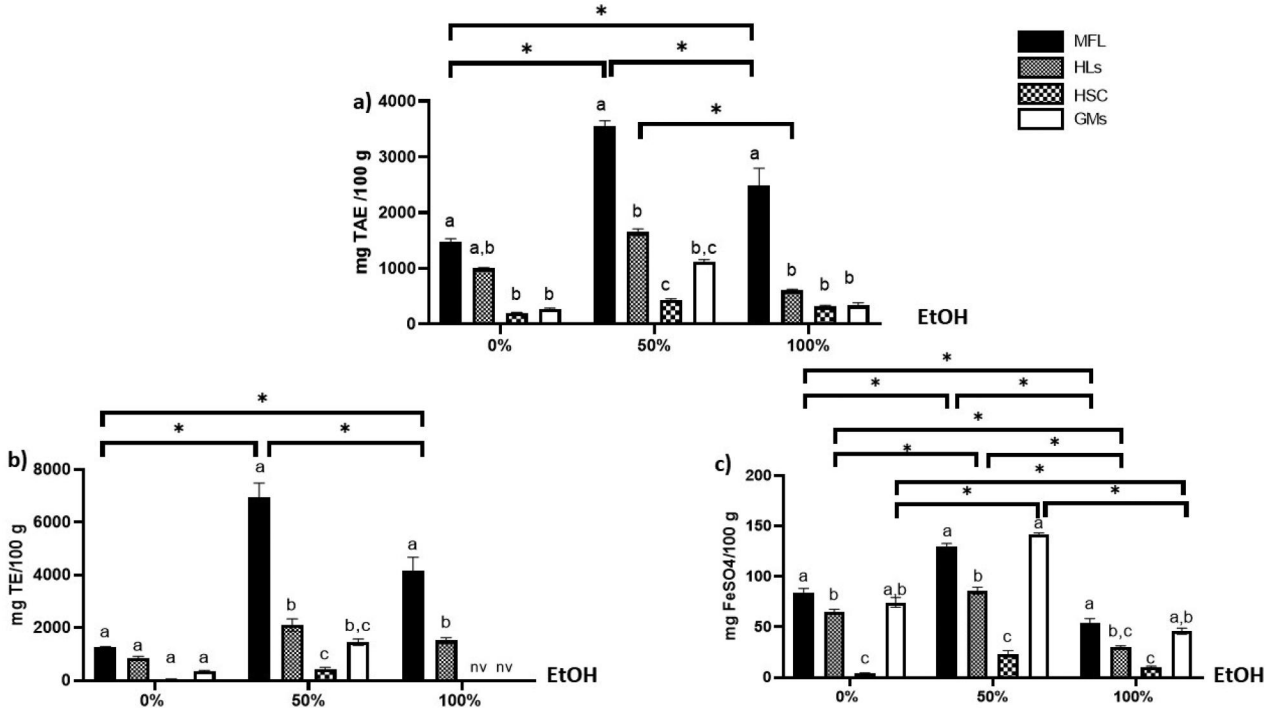

Figure 2. Total phenolic content and antioxidant activity (ABTS and FRAP) of green chemical extracts. TAE: Tannic acid Equivalent, TE: Trolox Equivalent. Data are presented as mean ± standard error of mean (SEM), ($n = 3$). Different superscript letters in columns indicate significant differences ($p < 0.05$) between the samples within each extraction. Bars indicate significant differences ($p < 0.05$) between the same samples in different extractions. (**a**) Total phenolic content; (**b**) ABTS assay; (**c**) FRAP assay.

in the hull than in the kernel fraction of HSs, and the dehulling process leads to a higher concentration of phenols (Chen et al., 2012).

Finally, as shown in Figure 2a, HSC showed no significant difference in each ratio tested. These results partially confirmed what was observed by Teh et al. (2014). More specifically, the authors reported a TPC for HSC, extracted with 100% EtOH, of 351.33 ± 2.08 mg GAE/100 g, highly comparable to that observed in this work (315.67 ± 22.42 mg TAE/100 g). With regard to GMs, on the other hand, binary extraction was not significantly different between 0% EtOH and 100% EtOH, although an upward trend was observed, partially confirming what was reported by Spigno et al. (2007).

Rotta et al. (2017) reported the importance of measuring the antioxidant property using at least two methods based on different reaction mechanisms. Therefore, as shown in Figures 2b and 2c, the antioxidant capacity was evaluated using the ABTS and FRAP assays. More precisely, Figures 2b and 2c show that the antioxidant activity presents the same trend as the TPCs, confirming the active role of phenolic compounds in antioxidant properties, as reported by Özbek et al. (2020) on pistachio HLs. At the same time, the authors demonstrated how concentrations between 70% and 100% EtOH reduced antioxidant capacity. In our study, this effect only occurred for MFLs in the ABTS assay, whereas for FRAP in all matrices except HSC. This distinction indicates that the type of bioactive compounds and the relative amounts recovered are influenced by the ratios of H_2O and EtOH. Indeed, with the polarity of the solvent varying from very polar (100% H_2O) to less polar (0% H_2O), the ability of the solvent to dissolve selected groups of antioxidants also varies and this has an implication on the subsequent antioxidant activity (Lim et al., 2019).

Thus, as reported, solvent extraction is the most common means of recovering phenolic compounds from plant-derived materials due to the simplicity of the technique, its efficiency, and the wide range of possible applications. Although, as reported in the literature, there are several solvents that can be used (methanol, hexane, acetone, ethyl acetate) (Sun et al., 2015), the possibility of combining EtOH with H_2O in any ratio, the different polarity of both solvents, and their acceptability for human consumption, make their mixtures the most suitable solvents to obtain phenolic extracts from plants, without leaving toxic residues (Conde-Hernandez and Guerrero-Beltran, 2014).

High-performance thin layer chromatography and dpph of green chemical extracts

Because of the interesting results obtained with the 50% EtOH extraction, the next step was to characterise the phenolic composition by HPTLC analysis.

In this study, HPTLC was used to semi-quantitatively characterise flavonoids, phenolic acids, CBG, and α-tocopherol content in the samples. The HPTLC analysis was also useful for assessing the contribution of various types of phenolic compounds (such as flavonols, flavan-3-ols and phenolic acids) to the overall antioxidant activity, which can be directly related to specific molecules in the samples.

As shown in Figure 3a, hemp co-products showed a complex and rich profile.

More specifically, the band patterns in the chromatographic run of the MFL and HLs samples showed a greater degree of similarity than those observed in the HSC and GMs samples. In particular, as shown in Figure 3b, MFL and HLs showed similar bands with same Ratio frontis (*Rf*) to those used for the standards, namely Quercetin (*Rf* = 0.79), Kaempferol (*Rf* = 0.83), Rutin (*Rf* = 0.16), Chlorogenic acid (*Rf* = 0.29), and Ferulic acid (*Rf* = 0.78) (*Rf* are shown in Table 2). These phenolic compounds are characterised by high antioxidant activity, as shown in Figure 3b, confirming the results reported by Izzo et al. (2020a), Nagy et al. (2019), and Aloo et al. (2023). At the same time, the MFL revealed matches for the α-tocopherol band, an isoform of vitamin E (*Rf* = 0.96), supporting the results shown by Beleggia et al. (2023). In particular, the authors, characterising the phytochemical profile of hemp flowers, detected the high presence of tocopherol. Of this, the main isoform present was α- (86.6–89.4%), followed by β + γ- (8.1–11.2%) and δ-tocopherol (1.5–2.3%) (Beleggia et al. 2023).

The presence of α-tocopherol was also found in HLs. As reported by Izzo et al. (2020b) and Engin (2009), this molecule is highly present in HSs oil where it covers a key role in preserving the oxidative stability of oils, acting as a chain-breaker, and consequently slowing down the lipoperoxidation process, confirming the results reported in Figure 3b. What has just been observed plays an important role in that, even following the dehulling process, the lipid profile in the endosperm of the HSs is not altered, thus improving the nutritional and functional profile of the HLs.

In parallel, MFL showed a match for CBG, an important phytocannabinoid, confirming the reports of Farinon et al. (2020) and Nagy et al. (2019). Phytocannabinoids are produced, collected, and stored at the level of pedunculated glandular trichomes,

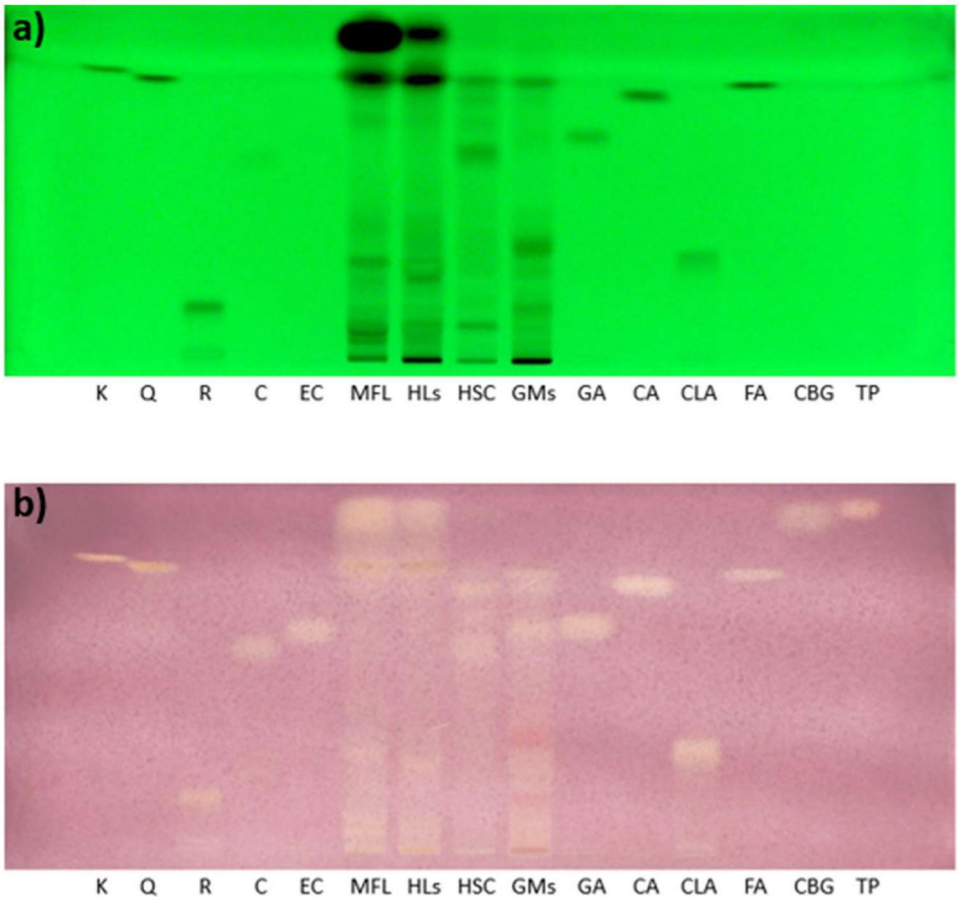

Figure 3. HPTLC plate of standards and samples detected 254 nm (a) and after derivatization with DPPH detected at visible light (vis) (b). K: Kaempferol, Q: Quercetin 3-O-glucoside, R: Ruthin; C: Catechin; EC: Epicatechin; GA: Gallic acid; CA: Caffeic acid; CLA: Chlorogenic acid; FA: Ferulic acid; CBG: Cannabigerol; TP: α-tocopherol; MFL: Mix flowers/leaves; HLs: Hulls; HSC: Hemp seed cake; GMs: Grape marcs.

Table 2. HTPLC standards with their correspondent *Ratio frontis* (Rf) and γ (nm) values. vis: visible light (380–700 nm).

Standard	Abbreviations	Ratio frontis (*Rf*)	γ (nm)
Kaempferol	K	0.83	254, vis
Quercetin 3-O-glucoside	Q	0.79	254, vis
Rutin	R	0.16	254, vis
Catechin	C	0.57	vis
Epicatechin	EC	0.62	vis
Gallic acid	GA	0.63	254, vis
Caffeic acid	CA	0.75	254, vis
Chlorogenic acid	CLA	0.29	254, vis
Ferulic acid	FA	0.78	254, vis
Cannabigerol	CBG	0.94	vis
α-tocopherol	TP	0.96	vis

small specialised secretory epidermal glands present and abundant on flowers, in fewer numbers on leaves and stems while they are absent at the level of seeds, so the latter organs should not contain cannabinoids (Farinon et al., 2020). However, HLs also showed bands for CBG. Most probably, this is caused by contamination with other parts of the plant during harvesting. In fact, as reported by Farinon et al. (2020), the presence of cannabinoids in HSs represents an accidental contamination due to both the considered cultivar and the seed cleaning process. Most probably, the confirmed presence of CBG, a highly antioxidant molecule as shown in Figure 3b, influenced the results previously reported with the ABTS and FRAP assays following chemical extraction for HLs, resulting in an increased antioxidant profile.

For HSC, bands were observed for Quercetin, Ferulic acid, Caffeic acid ($Rf = 0.75$), and Catechin ($Rf = 0.57$), partially confirming the results reported by Teh et al. (2014). More specifically, the authors reported the presence of Caffeic acid and Quercetin in HSC, but the absence of Ferulic acid and Catechin, although the latter, as shown in Figure 3b, is more evident following derivatisation with DPPH. As reported by Ingallina et al. (2020), the presence/absence and relative concentrations of phenolic compounds depend on environmental conditions and seasonality, resulting in different profiles even when considering the same matrix. These compounds are characterised by important antioxidant activity, as shown in Figure 3b, particularly Caffeic acid

and Catechin, confirming the results reported by Gülçin (2006) and Grzesik et al. (2018).

Total phenolic content and antioxidant activity of hemp co-products ex vivo digested

Figure 4 shows graphs of the TPC, ABTS, and FRAP of hemp co-products following *ex vivo* digestion.

As reported in Figure 4a, all the matrices analysed showed the same trend, characterised by an increase in TPC after the end of the gastric phase (2h), although it was only statistically significant ($p < 0.05$) for HLs and GMs. This trend is confirmed on matrices digested with *in vitro* digestion protocols (Goulas and Hadjisolomou, 2019; Ma et al., 2020; Lanzoni et al., 2023a). In general, as reported in the literature, the behaviour of phenols during the digestive process is highly influenced by both the food matrix and the digestion conditions (Olivas-Aguirre et al., 2017). More specifically, in the stomach, the acidic pH (2.0–3.0) allows the release of phenolic compounds from the food matrix following the breaking of protein and polysaccharide (fibre) bonds (Goulas and Hadjisolomou, 2019), increasing their availability, thus confirming the results obtained in this study. In parallel, as shown by Pineda-Vadillo et al. (2016), the acid pH protects phenols during gastric digestion, not altering their quantification. As shown in Figure 4a, at the end of the intestinal phase (4h), there was a decrease in TPC compared to the gastric phase, although statistically significant ($p < 0.05$) only for HLs. This trend is caused by the instability of phenolic compounds at the alkaline pH (>7.0), typical of the intestinal environment, especially during pancreatic action, which leads to the transformation of phenols into unknown structural forms with different chemical properties, different bioaccessibility, bioavailability, and biological activity (Wojtunik-Kulesza et al., 2020). Considering the individual matrices, interesting results are reported for HLs (486.57 ± 11.56 mg TAE/100 g) at the end of the gastric phase, with statistically ($p < 0.05$) higher differences compared to HSC (332.28 ± 17.10 mg TE/100 g) and GMs (291.68 ± 27.86). Although, dietary fibres are the main carriers of phenolic compounds, thus influencing their bioaccessibility, as reported by Wojtunik-Kulesza et al. (2020), it is also true that in HSs, as previously observed, the phenols are mainly localised in the HL, and following the gastric phase, the disintegration of the food matrix results in a high release, leading to a greater concentration than in other samples (House, 2021).

In parallel, as shown in Figures 4b and 4c, the ABTS and FRAP (with the exception of MFL) assays showed

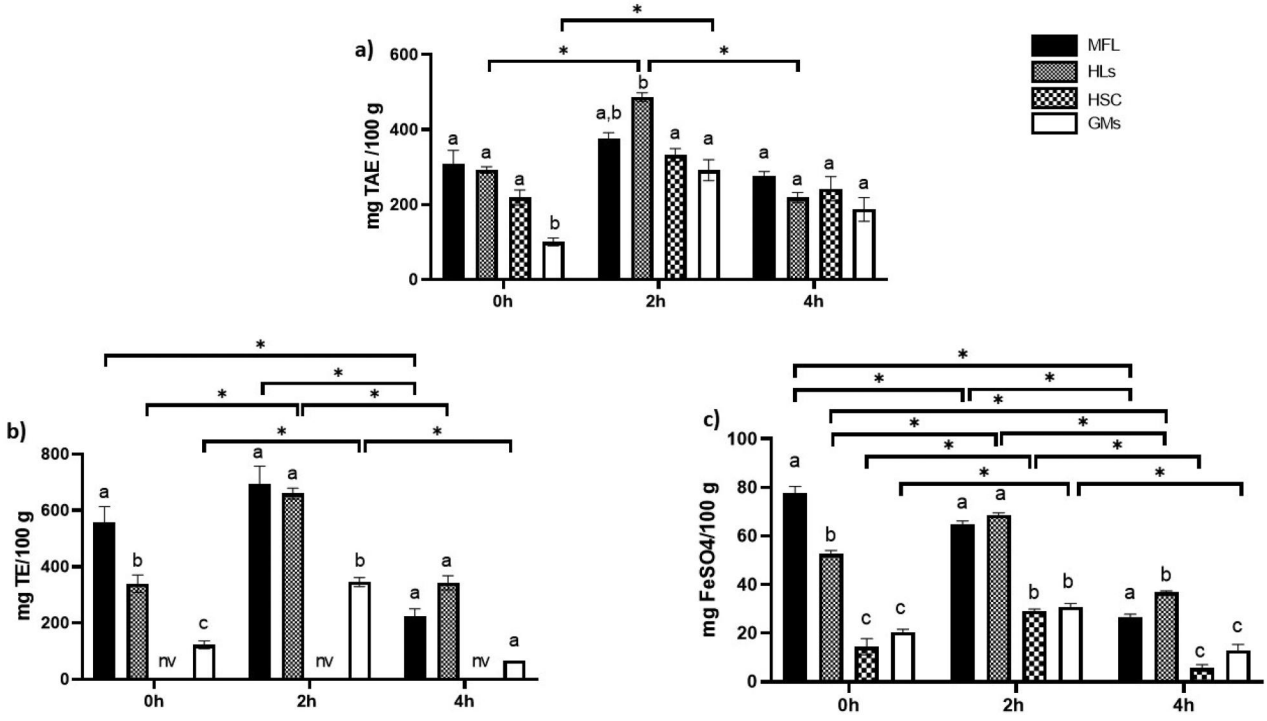

Figure 4. Total phenolic content and antioxidant activity (ABTS and FRAP) of *ex vivo* digestion of hemp co-products. TAE: Tannic acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± standard error of mean (SEM), ($n = 3$). Different superscript letters in columns indicate significant differences ($p < 0.05$) between the samples within each digestion phase. Bars indicate significant differences ($p < 0.05$) between the same samples in different digestion phase. (**a**) Total phenolic content; (**b**) ABTS assay; (**c**) FRAP assay. 0h: start of gastric phase; 2h: end of gastric phase; 4h end of intestinal phase.

the same trend as the TPC, confirming that phenolic compounds are actively involved in antioxidant activity, as reported above. As phenolic compounds, the antioxidant capacity is also influenced by digestion conditions. Wojtunik-Kulesza et al. (2020) reported that the low pH, typical of the gastric phase, increases the antioxidant power of the compounds due to an increase in their ability to donate electrons. In parallel, the decrease observed for each sample at the end of the intestinal phase could be attributed either to a structural reorganisation of some phenolic compounds due to their sensitivity to alkaline pH or to the fact that, during digestion, some of them are able to bind other constituents of the food matrix, forming complexes that inhibit their antioxidant power (Wojtunik-Kulesza et al., 2020). Considering the individual matrices, MFL and HLs showed statistically significant differences from HSC and GMS, with the exception of the ABTS assay at the end of the intestinal phase, confirming what was observed following the green chemical extraction. Although these results can be attributed to the high concentration of phenols, compounds such as α-tocopherol and CBG play a major role in functional activity, as shown before. At the same time, multiple biological functions are correlated with CBG, including anti-inflammatory, antibacterial, antifungal, redox balance regulation, and as a neuromodulator (Jastrząb et al., 2022). However, the presence of phytocannabinoids, particularly THC, despite their positive effects, raises concerns about possible bioaccumulation in animal products with a resulting health risk for the consumer. For this reason, it should be emphasised that the supplementation of hemp-based products in the diet of broilers did not result in traces of THC in the breast, liver, lungs, and thighs, confirming the safety of these compounds (Jing et al., 2017). As shown in Figure 4c, MFL showed a different trend at 0h, suggesting a ready availability of compounds with antioxidant capacity to chelate metals, the principle of action of the FRAP assay.

In Figure 4b, the values for HSC were not shown, as the ABTS assay obtained non-detectable values at each stage of the digestive process. This result could most likely be related to the behaviour of the phenols during digestion. As reported above, HSC is mainly characterised by the presence of caffeic acid, ferulic acid, quercetin and catechin. However, as reported by Wojtunik-Kulesza et al. (2020), not all phenols are able to perform the same antioxidant activity. More precisely, as shown by Khokhar and Apenten (2003), some conditions such as alkaline pH or the presence of oxygen can inhibit the antioxidant action and induce a pro-oxidant behaviour of some phenols, especially to the damages of small compounds, among them caffeic acid. In parallel, ferulic acid appears to be one of the main phenolic compounds linked to lignin and non-starch polysaccharides in HSs; factors that could inhibit its antioxidant action (Wojtunik-Kulesza et al., 2020). However, results obtained by FRAP assay showed highly comparable values with GMs at every stage of the digestive process. This result could be related to the fact that Ferulic acid, Caffeic acid, Quercetin, and Catechin are phenolic compounds also characterised by metal chelating antioxidant activity (Kashima, 1999; Gülçin, 2006; Leopoldini et al., 2006; Zduńska et al., 2018).

Hemp co-products were characterised by an adequate nutritional profile and a high functional potential. However, to date, the use of hemp-based products in the feed sector is limited, due both to the current regulatory status, the different processes on matrices, the cost and limited volumes produced (Salami et al., 2019; Vastolo et al., 2022). More precisely, as shown by Moscariello et al. (2021), the price of HSs, the main product of hemp for food and feed purposes, is around 1.33/1.99 €/kg with a production of 0.6-0.9 ton/ha per year, a price significantly higher than that of soybeans (0.42/0.50 €/kg) (Granaria Commodity Trading Association Milano, 2024), the main protein source used in the diet of livestock animals. However, although cost is a critical point, the interesting nutritional but above all functional profile of hemp-based products compared to soy products, as also demonstrated in our previous work (Lanzoni et al., 2023a), suggest their use in the diets of monogastric animals.

For these reasons, to stimulate the use of hemp-based products and their co-products, it is necessary to increase cultivation and production by creating new markets for hemp, thus ensuring the core principles of the Green Deal, One Health and F2F.

Conclusions

Hemp co-products showed an interesting nutritional profile, especially in terms of protein and lipids, comparable or superior to that of grape marcs, a matrix widely used in the feed industry. For HLs and MFL, valuable results were obtained in terms of TPC and antioxidant activity (ABTS, FRAP and DPPH), both following green chemical extraction and *ex vivo* digestion. Extraction with 50% EtOH showed to be the most effective for extracting phenolic compounds and antioxidants from plant-derived matrices. This work

represents a preliminary report on the functional profile of hemp co-products for their future application in the feed industry. *In vivo* trial will be necessary to evaluate their efficacy on the health and growth performance of farm animals, taking into account the high variability of these matrices. The presented data support the potential future growth of hemp processing industries, thus creating markets capable of absorbing large quantities of scraps and consequently valorising it into co-products for the feed industry.

Ethical approval

Not applicable

Disclosure statement

No potential conflict of interest was reported by the author(s).

Acknowledgement

The authors would like to thank the company Roero Green Flower, for the valuable advice and material provided for the research.

Funding

This study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscripts reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them. Part of the data was presented at the Feed International Congress (Milan, 9–10 October 2023).

ORCID

Davide Lanzoni http://orcid.org/0000-0002-8233-659X
Francesca Mercogliano http://orcid.org/0000-0002-1785-0437
Raffaella Rebucci http://orcid.org/0000-0002-0731-2408
Corinne Bani http://orcid.org/0000-0002-9736-8746
Luciano Pinotti http://orcid.org/0000-0003-0337-9426
Chiara Di Lorenzo http://orcid.org/0000-0002-2798-8720
Giovanni Savoini http://orcid.org/0000-0002-8377-9376
Antonella Baldi http://orcid.org/0000-0002-5543-2455
Carlotta Giromini http://orcid.org/0000-0002-3717-5336

Data availability statement

The dataset generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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