

Study on the NIR Spectroscopy to predict energy content of Hemp flour (*Cannabis sativa* L.)

ABSTRACT

Aims: The aim of this study was to evaluate the utility of NIR_S for predicting the energy content through the chemical characterization of the flour obtained after the cold pressing of *Cannabis sativa* L. seeds, as well as the possibility of predicting their energy content starting from the data obtained through the NIRs technique.

Study design: The chemical composition of 56 hemp flour samples was determined following the official protocols of the Association of Analytical Chemists and chemometric readings were conducted. GE, gross energy digestibility (GE_d) and digestible energy (DE) were estimated using the equations proposed by INRA. A statistical analysis was performed to evaluate the potential use of NIR data to predict the energy content of hemp flour.

Results: Data from laboratory and NIR assessments were 22.54 versus 20.44 for GE (MJ/kg DM), 90.72 versus 90.21 for GE_d (MJ/kg DM), and 19.73 versus 20.13, respectively for the loss (%). The results indicated the feasibility of energy value prediction, although further studies are needed to refine the technique. NIR expands the calibration set, allowing increasingly accurate determinations, in the study of the chemical-nutritional characteristics of hemp sativa, even if further investigations are necessary.

Conclusion: The study provides comprehensive insights into the chemical composition of hemp flour, explores its comparison with other seeds, evaluates different analysis methods, and establishes reliable prediction models for energy content.

Keywords: gross energy, gross energy digestibility, digestible energy, Hemp flour, near-infrared spectroscopy

1. INTRODUCTION

Animal nutritionists have long recognized the importance of measuring the nutritive value of feed provided to livestock. The nutritional value of hemp seeds and their processed products, such as oil and flour, has been studied in recent years to understand the nutritional quality of this food matrix [1,2]. Hemp, traditionally used as a source of fiber, is now increasingly considered for animal feed due to its favorable nutritional characteristics.

The literature indicates that hemp holds promise as a viable alternative to soybeans. This is attributed to hemp sharing essential characteristics with soybeans as a rotational crop, including profitability, potential as an energy crop, and the ability to maintain soil fertility. The findings reveal observable patterns of both complementary and substitution relationships in the case of hemp–wheat and hemp–soybean pairings, respectively. Moreover, the results suggest the potential for hemp monoculture to exhibit a positive response, enduring for multiple years, in reaction to self-positive shocks affecting hemp acreage [3]

Hemp flour, and specifically hemp seeds, contain high levels of omega-3 and omega-6 fatty acids. If hemp were incorporated into the diet of livestock, it could enhance the fatty acid

33 content in the final products derived from these animals. Consequently, the consumption of
34 these enriched products may also yield health benefits for humans.

35 The conducted research has revealed that the use of hemp seeds or their by-products as a
36 supplement in the diet of dairy ruminants promotes an improvement in the fatty acid profile of
37 the milk they produce. Particularly in dairy cows, an increase in urea concentration has been
38 observed, attributed to the rise in the concentration of raw proteins in the diet, along with a
39 decrease in fat and protein content in the milk. Unfortunately, this study did not maintain an
40 equivalent concentration of proteins and fats in the various diets used to feed the cows, and
41 there was no assessment of the acidic profile of their milk [4]

42 Experiments on digestibility of hemp seed flour have also been conducted in both sheep and
43 cows, indicating that the flour is as digestible as canola flour [5]. As expected, the seeds of
44 the plant represent the most nutritious fraction, with an average crude protein value of
45 around 21.77% of dry matter and an average lipid content of 23.5% of dry matter [4].

46 Hemp seed production data for 2020 are available from five countries in the FAO database
47 [6]. In Italy, the cultivation of industrial hemp has been permitted through law no. 242/2016,
48 along with the subsequent ministerial circular published in 2017, which outlines the
49 conditions for hemp production, marketing, and use [7].

50 There are various microscopic techniques used for sample analysis, including scanning
51 electron microscopy (SEM), transmission electron microscopy (TEM), and infrared
52 spectrophotometry. In scanning electron microscopy (SEM), the emitted electronic beam is
53 controlled to perform a television-type scan, exploring the surface of the object under
54 examination.

55 The transmission electron microscope (TEM) shares a schematic structure with an optical
56 microscope, replacing the light source with an electron source and using electromagnetic
57 lenses instead of optical ones. The interior of the electron microscope operates under
58 vacuum conditions. The electron source generates a beam of electrons with uniform velocity,
59 concentrated onto a thin film of the sample being observed. After passing through the
60 sample, the electron beam encounters the magnetic fields of the objective and projector,
61 reach a fluorescent screen to produce a visible image or a photographic plate.

62 The infrared spectrophotometry, instead, involves recording interactions between materials
63 and infrared radiation, inducing molecular vibrations associated with different functional
64 groups. By interpreting spectroscopic signals acquired through spectrum acquisition, it
65 becomes possible to identify substances or families of substances that generated the
66 "infrared spectrum."

67 Infrared imaging has considerable advantages over conventional mapping experiments,
68 namely short measuring times and improved spatial resolution. As with other microscopic
69 techniques, the output of the measurements is readily comprehensible to non-
70 spectroscopists.

71 The increased use of near-infrared reflectance spectroscopy (NIR_S) as an alternative to
72 traditional analytical methods for evaluating the energy content of feedstuffs and diets has
73 led to an expansion of knowledge in the field of chemometrics. NIR_S is a non-destructive,
74 fast, accurate, and less expensive technique for estimating the chemical composition of
75 feedstuffs [8]. Additionally, NIR_S offers advantages over conventional laboratory analytical
76 methods, such as no reagent use and simultaneous determination of multiple parameters
77 (e.g., crude proteins, ether extract, acid detergent fiber, neutral detergent fiber, etc.).

78 Like classic methods, drying and grinding procedures are fundamental for the NIRS
79 technique.

80 Water strongly absorbs NIR light, and particle size influences the shape of the spectrum.
81 NIR_S spectra are also affected by laboratory conditions (e.g., environmental dampness and
82 temperature), which should be as uniform as possible, particularly with respect to
83 temperature [9].

84 A lack of comprehensive data on the availability of various nutrients in feedstuffs and feeds
85 has hindered the use of NIR_S for estimating nutrient content for many animal species and

86 estimating energy content for ruminants [10, 11]. Hemp flour, obtained after pressing oil from
87 the seeds, is an exceptional raw material for producing products with a high nutritional
88 profile. Its value lies in its nutritional composition, characterized by a high content of protein,
89 fiber, and fats, along with vita-mins E, B1, and B2, mineral salts, and phytosterols.
90 Importantly, it does not contain gluten, making hemp flour ideal for preparing products
91 suitable for people with celiac dis-ease. Additionally, it is used in animal nutrition as a protein
92 source, replacing flours from more common oil seeds (soybean, rapeseed, and sunflower).
93 For animal feed purposes, the high content of NDF provides the flour with a quantity of
94 digestible principles not exceeding 40%, resulting in reduced digestibility. Despite the lower
95 digestibility, hemp flours have a moderate content of digestible protein (about 80%), making
96 them suitable for the feeding of some animal species, such as sheep, goats, and horses,
97 and less suitable for feeding pigs. Some studies report the benefits of using hemp cake for
98 feeding laying hens due to the presence of omega-3 and omega-6 fatty acids in the eggs
99 produced and as feed for farmed fish [12].
100 However, few studies have been published on the use of NIR_S to assess the composition
101 and nutritive value of food, possibly due to the difficulty in obtaining in vivo data for a robust
102 calibration.
103 The aim of this study was to evaluate the utility of NIR_S for predicting the energy content
104 through the chemical characterization of the flour obtained after the cold pressing of
105 *Cannabis sativa L.* seeds, as well as the possibility of predicting their energy content starting
106 from the data obtained through the NIRs technique.

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109 2. MATERIAL AND METHODS

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111 A set of 56 hemp samples, specifically the Futura75 variety, was obtained from two different
112 farms located in the Campania Region (Southern Italy). After collection, the samples were
113 ground using a 1 mm sieve with a knife mill and subjected to chemical composition analysis,
114 including dry matter (DM), crude protein (CP), ethereal extract (EE), and ash. The analysis
115 was conducted according to the procedures outlined by [11], with the respective
116 identification numbers 2001.12, 978.04, 920.39 and 930.05 assigned to DM, CP, EE and
117 ash. Furthermore, the neutral detergent fiber, acidic detergent fiber and acidic detergent
118 lignin of the free ash were determined, following the guidelines provided by [13].

119 From the rough analysis, gross energy (GE), gross energy digestibility coefficient (GE_d) and
120 digestible energy (DE) were estimated using the equations proposed by [15] and
121 researchers from [16].

122 Fresh food samples were ground through a 1 mm sieve and scanned twice in reflectance
123 mode in the spectrophotometer using a Büchi instrument (model NIRFlex N-500 Inc). The
124 NIRS spectrometer works in the near infrared spectral region (12500-4000 cm⁻¹). It consists
125 of a halogen lamp as a source and an array of InGaAs diodes and an intense broadband
126 light source, which allows the measurement of reflectance from a large area of the sample
127 surface (in a container of approximately 10 cm in size diameter). The diodes were centered
128 at 10 nm intervals, but software was used to interpolate the spectra over a 5 nm data
129 interval. The instrument's two spectral ranges are joined at 950 nm to cover a range from
130 800 to 2500 nm, chosen because many absorptions characteristic of amines fall in the same
131 regions as alcohols where the N-H and OH bonds are similar.

132 The analysis was carried out in reflectance to minimize the effects of the physical shape of
133 the sample.

134 The acquisition time of the instrument averaged 30 spectra s⁻¹ and a spectral scan was
135 defined as the average spectrum generated after 1 s of acquisition.

136 It should be noted that hydrogen bonds occur at very high frequencies due to the very low
137 mass of this atom. This is why the intensity of the transmitted radiation was measured in the
138 near-infrared (NIR) field rather than in the mid-infrared (MIR).

139 The chemical composition and measured GE, GEd and DE of the compound feeds used for
 140 calibration are presented in Tables 1 and 2.
 141 All statistical methods for data evaluation were performed to determine the possible use of
 142 NIR data to predict the energy content of hemp flour extraction and the relationships
 143 between all data considered using [17].

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146 3. RESULTS AND DISCUSSION

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148 In Table 1, it is observed that the flour is rich in protein using both methods. The NDF
 149 represents a high proportion in hemp flour. Table 1 shows lower protein content and higher
 150 structural carbohydrate content compared to the literature [12, 18, 2]. These differences
 151 could be attributed to the oil extraction method, which may have influenced the chemical
 152 composition of the analyzed samples. Similar results were reported by [18], who tested
 153 hempseed cake in cattle nutrition. The table also shows a high concentration of NDF in the
 154 cold-pressed hemp seed cake. The fat content may be justified by the pressing process,
 155 which removes approximately 63% of the fat from the whole seed, as indicated by [19].

156 If we compare the protein content obtained in our analyses, the data show that hemp has a
 157 higher protein content than seeds such as rapeseed and sunflower but lower than soy. In
 158 fact, soybeans are considered the main source of vegetable proteins, with a composition
 159 very close to that of animal-origin food. It should be noted that while soybeans contain anti-
 160 nutritional factors, such as trypsin inhibitors requiring thermal treatment for elimination, hemp
 161 contains a smaller amount, making its proteins more digestible [20].

162 The significant differences shown in Table 1 are related to the different methods used for
 163 determination. In particular, the NIRs technique involves more reading replications of a
 164 single sample compared to the duplication that takes place in the laboratory. Even though
 165 the reliability of the results in NIRs depends a lot on the calibration curves that need
 166 continuous updating, the differences, except for the ash content compared to NFE, are not
 167 significant enough to deem spectroscopy readings unreliable.

168 Table 1 - Chemical composition obtained from traditional chemical composition and NIRs
 169 data on hemp flour samples.

		Min	Max	Means	Std dev
Weende	DM	91.02	94.32	93.20	0.76
Van	CP	19.82	26.89	23.82**	1.53
Soest	NDF	48.94	62.87	56.18	3.50
	EE	7.10	27.87	12.09**	5.99
	ASH	5.14	8.49	7.03*	0.80
NIRs	DM	88.66	98.38	93.98	2.86
	CP	10.12	24.76	20.19**	3.63
	NDF	34.20	59.62	47.28	7.01
	EE	5.04	19.52	10.68**	2.86
	ASH	2.88	5.86	3.90*	0.75

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171 Table 2 - GE content, apparent digestibility of GE and DE content obtained from traditional
 172 chemical composition and NIR data on hemp flour samples.

Min.	Max	means	std.	Dev.
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	GE	20.7328	3722.54	1.70
Weende	GE _d	89.7	93.0 90.7	0.90
	DE	18.7626	2919.73	1.49
	GE	19.5825	2220.44	1.72
NIR	GE _d	88.3	91.8 90.2	1.70
	DE	17.3123	1220.13	1.18

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The data shown in Table 2 demonstrate good correspondence between the data calculated from chemical determinations (Weende and Van Soest) and those obtained with NIRs determinations. The apparent digestibility of GE values obtained appears high. [20] and [21] report that hemp flour has high digestibility related to a high degree of digestibility of proteins. [20] reports that this good digestibility can be linked to an immediate release of bio accessible amino acids.

In Table 3 we report the correlation matrix among the considered parameters.

Table 3 - correlation matrix among the considered parameters

	Correlations	Sign.
GE vs GE_NIR	-0.035	NS
GE _d vs GE _d _NIR	-0.317	0.017
DE vs DE_NIR	-0.141	NS

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In Table 3, we report the correlation matrix among the considered parameters. No significant correlation was found between GE and DE. However, for all parameters obtained by NIRs techniques, a negative correlation was found. These results are probably related to the different values obtained in the chemical compositions and reported in Table 1.

The data were also analyzed separately for GE, GE_d, and DE using multiple linear regressions to evaluate the relationship between energy content and various predictor variables. Stepwise regression was used to eliminate variables that did not influence variation in the model. The R²_{adj} selection method was used to make the final decision about the best models.

From the examination of regressions (Table 4 and 5), it is possible, using CP_NIR, EE_NIR, NDF_NIR, and NFE_NIR as independent variables, to obtain a good prediction model for GE. Tables show that the best predicting model was the last one, as demonstrated by the best R²_{Adj} and the significant level of independent variables.

Table 4 - Linear regression model summary for GE prediction

Model	R	R ²	R ² _{Adj}	standard error of estimate
1	0.641a	0.410	0.399	1.314
2	0.723b	0.523	0.504	1.194
3	0.755c	0.571	0.545	1.144
4	0.977d	0.955	0.952	0.373

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- a. predictors: (constant), CP_NIR
 b. predictors: (constant), CP_NIR, EE_NIR
 c. predictors: (constant), CP_NIR, EE_NIR, NDF_NIR
 d. predictors: (constant), CP_NIR, EE_NIR, NDF_NIR, NFE_NIR

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Table 5 - ANOVA for the regression models d

Model	sum of squares	df	Mean Square	F	Sign.
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Regression	145.51	4	36.38	261.60	0.0001
Residual	6.81	49	0.14		
Total	152.32	53	36.38		

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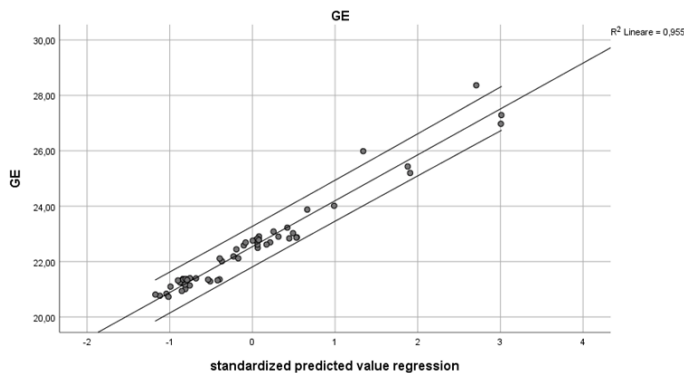
Examination of the table shows the goodness of the forecast model chosen, as highlighted by the low value of the residue. The predictive model summary for evaluating GE shows high R^2 and R^2_{Adj} values.

GE may be predicted with the following equation:

$$Y = -0.14 - 0.377(CP_NIR) + 0.744(EE_NIR) + 0.323(NDF_NIR) + 0.328(NFE_NIR) \pm \epsilon$$

In Figure 1, we report the relationship between observed and predicted GE with the confidence interval at 95% obtained with the linear regression model. The distribution of the standardized residuals of the predicted regression value confirms the correctness of the model applied to our hemp flour sample.

Figure 1 - The relationship between GE observed and GE predicted for the statistical model.



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Tables 6 and 7 show the linear regression model summary for GEd prediction and the ANOVA for the regression models.

Table 6 - Linear regression model summary for GEd prediction

Model	R	R^2	R^2_{Adj}	standard error of estimate
1	0.660a	0.436	0.426	0.73402
2	0.894b	0.798	0.791	0.44305
3	0.919c	0.845	0.836	0.39172

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- a. predictors: (constant), CP_NIR
- b. predictors: (constant), CP_NIR, EE_NIR
- c. predictors: (constant), CP_NIR, EE_NIR, ASH_NIR

The predictive model summary for evaluating GEd shown high R^2 and R^2_{Adj} values.

Table 7 - ANOVA for the regression models c

Model	sum of squares	df	Mean Square	F	Sign.
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Regression	43.627	3	14.542	94.772	0.001
Residual	7.979	52	0.153		
Total	51.606	55			

236 Examination of the table shows the goodness of the forecast model chosen, as highlighted
 237 by the low value of the residue.

238 GED may be predicted with the following equation:

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240 $Y = 92.65 - 0.352(\text{CP_NIR}) + 0.348(\text{EE_NIR}) + 0.375(\text{Ash_NIR}) \pm \epsilon$

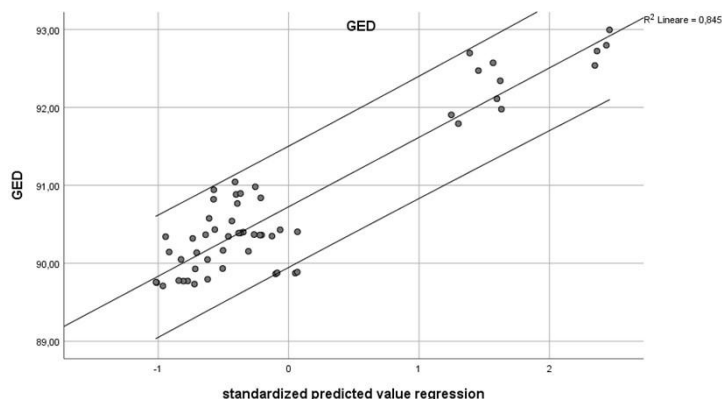
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242 In Figure 2, we report the relationship between observed and predicted GEd with the
 243 confidence interval at 95% obtained with the linear regression model. The distribution of the
 244 standardized residuals of the predicted regression value confirms the correctness of the
 245 model applied to our hemp flour sample.

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247 Figure 2 - The relationship between GEd observed and GEd predicted for the statistical
 248 model.

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252 Table 8 and 9 shows linear regression model summary for DE prediction and the ANOVA for
 253 the regression models.

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255 Table 8 - Linear regression model summary for DE prediction

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Model	R	R ²	R ² _{Adj}	standard error of estimate
1	0.659a	0.434	0.423	1.30956
2	0.760b	0.578	0.561	1.14214
3	0.786c	0.617	0.594	1.09788
4	0.974d	0.948	0.944	0.40926

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- a. predictors: (constant), CP_NIR
- b. predictors: (constant), CP_NIR, EE_NIR
- c. predictors: (constant), CP_NIR, EE_NIR, NDF_NIR
- d. predictors: (constant), CP_NIR, EE_NIR, NDF_NIR, NFE_NIR

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The predictive model summary for evaluating DE shown high R² and R²_{Adj} values.

265 Table 9 - ANOVA for the regression models d.
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	sum of squares	df	Mean Square	F	Sign.
Regression	149.297	4	37.324	222.841	0.001
Residual	8.207	49	0.167		
Total	157.504	53			

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268 Examination of the table shows the goodness of the prediction model chosen, as highlighted
 269 by the low value of the residue.

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271 DE may be predicted with the following equation:

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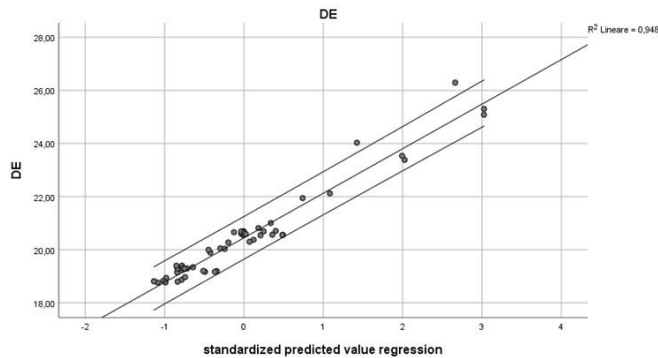
273 $Y = 0.10 - 0.436 (CP_NIR) + 0.773 (EE_NIR) + 0.304 (NDF_NIR) + 0.309 (NFE_NIR) \pm \epsilon$

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275 Figure 3 we report the relationship between observed and predicted DE with the confidence
 276 interval at 95% obtained with linear regression model. The distribution of the standardized
 277 residuals predicted regression value confirms the correctness of the model applied in our
 278 hemp flour sample.

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280 Figure 3 - The relationship between DE observed and DE predicted for the statistical model.
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284 Hemp flour shows protein richness using both methods, with a notable presence of NDF
 285 [21].

286 The protein content is in line with literature values, but the differences in protein and
 287 carbohydrate content compared to other studies can be attributed to the oil extraction
 288 method [22].

289 Differences in protein and carbohydrate content, in particular a higher structural
 290 carbohydrate content than literature values, may be linked to the influence of the oil
 291 extraction method on the sample composition [23].

292 The protein content of hemp is higher than that of rapeseed and sunflower, but lower than
 293 that of soy.

294 Although soy is a primary source of plant protein, hemp protein is more digestible due to
 295 fewer antinutritional factors [24].

296 Table 1 reveals significant differences attributed to the different methods, particularly with
 297 the NIR technique involving more read replicates than laboratory duplication.

298 Despite differences, the reliability of NIRs results is indicated, especially for parameters
299 except for ash content compared to NFE.
300 Table 2 demonstrates good correspondence between chemical determinations and NIRs
301 determinations, with apparent high digestibility of GE values.
302 No significant correlation was found between GE and DE, but negative correlations were
303 observed for all parameters obtained by NIRs techniques.
304 Multiple linear regressions were conducted, and the best predicting model for GE was
305 identified using CP_NIR, EE_NIR, NDF_NIR, and NFE_NIR as independent variables.
306 The predictive model for GE includes CP_NIR, EE_NIR, NDF_NIR, and NFE_NIR,
307 demonstrating high R² and R²Adj values.
308 GEd and DE are also predicted using similar equations, showing good model
309 correspondence and reliability.
310 Figures 1, 2, and 3 visually represent the relationship between observed and predicted
311 values, confirming the correctness of the applied models for GE, GEd, and DE.

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313 **4. CONCLUSION**

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315 The classic measurements of the chemical composition of food content using AOAC
316 methods require time and involve the use of solvents that must then be disposed of as
317 special waste. Near-Infrared (NIR) spectroscopy is rapid and does not produce special toxic
318 waste; therefore, it is being studied as a potential screening method for the analysis of
319 chemical composition. The study provides comprehensive insights into the chemical
320 composition of hemp flour, explores its comparison with other seeds, evaluates different
321 analysis methods, and establishes reliable prediction models for energy content.

322 **COMPETING INTERESTS**

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324 Authors declare that no competing interests exist.

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326 **AUTHORS' CONTRIBUTIONS**

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328 Conceptualization, F.S.; formal analysis, F.S. and G.P.; X.X.; investigation, G.A. and G.G.;
329 data curation, G.A. and R.P.; writing—original draft preparation, F.S. and G.A.; writing—
330 review and editing, F.S..

331 All authors have read and agreed to the published version of the manuscript.

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