CIENCIA DEL SUELO

METHODS TO DETERMINE NITROGEN IN SUNFLOWER GRAINS

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ABSTRACT

In sunflower, grain oil concentration (O_G) determines oil industrial yield and protein concentration (P_G) determines the protein of by-products as pellets and meals (P_M). There are various methodologies to quantify total grain nitrogen concentration (N_G): a) Kjeldahl (wet digestion); b) Dumas (dry combustion); c) NIRS (analysis of the spectrum of the light reflected by the sample). NIRS represents a promissory method for determining N_G because it is simple and fast. The objective of this work was to compare the methods of NIRS, Kjeldahl and Dumas to quantify N_G in sunflower grains and to adjust models to estimate P_M from N_G. Eighty-four samples of sunflower grains were selected covering a wide range of N_G (from 1.75 to 3.80% taking Dumas reference) from a net of experiments conducted in southeastern Buenos Aires Province (2013-2014 and 2014-2015). NIRS was the most precise method for the determination of N_G and presented a greater degree of agreement with Dumas than Kjeldahl. P_M was satisfactorily estimated with N_G. Therefore, NIRS could be a better alternative to the other techniques because its low cost, quickness, low toxicity, and adaptability to determine other components of the grain simultaneously with the determination of N_G.

Keywords: Dumas, Kjeldahl, NIRS, protein.

MÉTODOS PARA DETERMINAR NITRÓGENO EN GRANOS DE GIRASOL

RESUMEN

En girasol, la concentración de aceite (O_G) determina el rendimiento industrial de los granos y la concentración de proteína (P_G) determina la calidad de los subproductos como pellets y harinas (P_M). Existen varias metodologías para cuantificar la concentración de nitrógeno total en grano (N_G): a) Kjeldahl (digestión húmeda); b) Dumas (combustión seca); y c) NIRS (análisis del espectro de la luz reflejada por la muestra). NIRS representa un método promisorio para determinar N_G porque es simple y rápido. El objetivo de este trabajo fue comparar los métodos de NIRS, Kjeldahl y Dumas para cuantificar N_G en granos de girasol y ajustar modelos para estimar P_M a partir de N_G. Se seleccionaron ochenta y cuatro muestras de granos de girasol que cubren un amplio rango de N_G (de 1.75 a 3.80% tomando la referencia del método de Dumas) de una red de experimentos realizados en el sudeste de la provincia de Buenos Aires (2013-2014 y 2014-2015). El NIRS fue el método más preciso para la determinación de N_G y presentó un mayor grado de ajuste con Dumas respecto a Kjeldahl. La P_M se estimó satisfactoriamente con N_G. Por lo tanto, NIRS podría ser una alternativa superadora a las otras técnicas debido a su bajo costo, reducido tiempo

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de análisis, es no tóxico y puede determinar otros componentes del grano de manera simultánea con la determinación de N_G.

Palabras clave: Dumas, Kjeldahl, NIRS, proteína.

ABBREVIATIONS

N: nitrogen

N_G: total grain nitrogen concentration

O_G: grain oil concentration

P_G: grain protein concentration

P_M: meal protein concentration

INTRODUCTION

Nitrogen (N) is the main nutrient affecting yield and grain quality in sunflower (*Helianthus annuus* L.) (Alberio *et al.*, 2015). An efficient N management could help to reduce yield and quality gaps. However, N fertilization is not a common practice for farmers (Hall *et al.*, 2013). In addition, N plays an important role in determining grain oil concentration (O_G) and protein concentration (P_G) (Alberio *et al.*, 2015). From a commercial point of view, O_G determines oil industrial yield and P_G determines the quality of by-products as pellets and meals (Dauguet *et al.*, 2016). Although an adequate N availability is necessary to obtain grains with high O_G and P_G, excessive concentration of this nutrient could reduce O_G (Alberio *et al.*, 2015). However, our previous work showed a positive effect on N_G and, therefore, on P_G without decreasing O_G even with N rates up to 160 kg N ha^{-1} (Diovisalvi *et al.*, 2018). Therefore, it is essential to determine not only O_G but also N_G. There are various methodologies to quantify N_G. One of the first methods used is the Kjeldahl method (Jones Jr, 1991), which is based on the digestion of the sample with sulfuric acid and catalyzers at > 250 °C. Then, the distilled product is titrated with a solution of standardized sulfuric acid to determine N content. The disadvantages of this method are that it requires corrosive and-or toxic products, takes a long time, and its several steps can lead to errors (Jung *et al.*, 2003).

The Dumas method is an alternative to Kjeldahl method because it is faster (less than five minutes per sample), precise, and it can be semi-automatic and avoids using corrosive or toxic products, being a relative secure method (Jung *et al.*, 2003). It consists of a dry combustion of the sample at 950 °C (converting all N forms into N oxides and reduction to gaseous N), and then the conductivity is measured with a TruSpec CN analyzer (LECO, 2021). Several works analyzed the effectivity of the Dumas method compared with to Kjeldahl method. Only in cases with high nitrate concentrations, the analysis by Dumas produced higher N_G values because this method measures some N forms not quantified by the Kjeldahl method (Simonne

et al., 1997). Moreover, for soybean protein meals, cereal grains and dairy products both methods presented similar behavior. However, in most cases, laboratories use the Kjeldahl method as it is cheaper than the Dumas one (Jung *et al*. 2003).

Near infrared spectroscopy (NIRS) represents other method for determining N_G. This technique is widely used for the analysis of different traits of food quality and farm products because it is non-destructive, fast, and simple, and it allows working with a sample size more representative than in other methods (Batten, 1998). The NIRS technique is based on the analysis of the spectrum of the light reflected by the sample (wavelength 1100-2500 nm), which provides information of the product composition (Murray, 1993; Batten, 1998). It is an indirect or secondary method because it requires calibration with reference samples estimated with other method (chemical or physical) (Sáez-Plaza *et al.*, 2013), so it depends on the accuracy of the reference method, even though its precision may be superior (Batten, 1998). Under correct use, NIRS can be more profitable than traditional methods of wet chemistry (Manley, 2005). In sunflower, it was not developed as in other grains mainly because the spectrum of the hull does not represent the whole grain composition (Sato *et al.*, 1995; Perez-Vich *et al.*, 1998). However, calibrations for estimating O_G and fatty acid composition were developed with milled grains using this technique (Velasco *et al.*, 2004), but there is limited information about determining N_G in those grains. In addition, with NIRS technology no chemical products are used as in the Kjeldahl method and the cost per sample is lower than Dumas method. For these reasons, NIRS represents and interesting alternative to be used as a routine method in routine analysis (Velasco *et al.*, 2004).

Knowing P_G is useful to estimate protein in by-products (P_M) (González-Pérez, 2015; Dauguet *et al.*, 2016). Differences up to 1-2% in P_G could represent differences up to 5% in P_M, so it is essential to obtain grains with high P_G (Merrien *et al.*, 1988; Diovisalvi *et al.*, 2018). In the by-products market, pellets with PM> 36% have better prices than with lower protein (De Figueiredo *et al.*, 2015, Dauguet *et al.*, 2016). According to Dauguet *et al*. (2016), with P_M of 36%, prices would be equivalent to 70% of the price of soybean pellets, while at 29% of P_M, prices were equivalent to just 43%. Thus, it is essential to have methods to accurately determine N_G.

The objective of work was to compare the methods of NIRS, Kjeldahl and Dumas to quantify N_G in sunflower grains and to adjust models to estimate P_M from N_G.

MATERIALS AND METHODS

Genetic material and experimental design

Eighty-four samples of sunflower grains were selected from experiments conducted during the 2013-14 and 2014-15 growing

seasons in the southeastern Buenos Aires Province (from 37°45´ S, 58°17´ W to 38°40´ S, 60°08´ W). The experiments included 14 sites and 5 N rates (0, 60, 90, 120 and 150 kg N ha^{-1}) applied as surface-broadcasted urea at crop emergence in each site (**Table 1**). All sites, with different farming histories, were conducted under no-tillage system. Predominant soils are Petrocalcic Argiudoll (series fine, mixed, thermic) and Typic Argiudoll (series fine, mixed, thermic) (Soil Taxonomy) (United States, 1975) with a slope < 2%. The characteristics of this region are: mean annual rainfall of 955 mm, potential evapotranspiration of 950 mm, and mean temperature of 13.9°C. Different conventional and high oleic hybrids were sown (**Table 1**), but with similar characteristics: high yield potential and O_G, resistant to lodging, and good behavior to diseases (ASAGIR, 2021). Planting date were within the recommended ones for each site. The experimental design was a randomized complete block with three replications. Soil analysis before sowing, as reported at Diovisalvi et al. (2018), is shown in Table. (**Table 1**).

Sunflower grain samples were dried in an oven at 50ºC during 24 h, milled (particle size < 1mm), and homogenized. Milled samples were dried at 60ºC until constant weight for N_G analysis. The results of N_G were expressed in dry basis.

Determination of N_G with Kjeldahl method

Dry and milled sample (0.1 g) was placed in a 70 mL test tube, and 1.1 g of catalyzer and 4 mL of concentrated H_2SO_4 were added. The mixture heated in a digester (270ºC) until the sample became light green (approx. 1 h and 45 minutes). Once cold, the tubes were made up to 50 mL with distilled water and mixed. Ten mL NaOH at 45% were added to an aliquot of the sample and the mix was distilled. The distilled (35-40 ml) was recovered on 5 mL of H_3BO_3 indicator solution. Finally, it was titrated with H_2SO_4 0.005 N until the sample shifted from green to pink. The N concentration was calculated as follow:

N_G (%) = ((M-B) ml x 0.005 x 14 x 50 x 100) / (1000*10*0.1)

where M and B are milliliters (ml) of H_2SO_4 used in the titration of the sample (M) and the control (B); 0.005 is the normality of H_2SO_4; 14 is the equivalent weight of the N; 50 is the volume of the sample; 10 is the distilled volume; and 0.1 is the sample weight.

Determination of N_G with Dumas method

Dry and milled sample (0.2 g) was burned at 950ºC using high purity oxygen (99.9%). The product of this reaction was filtered, dried and quantified using an infrared cell. This procedure was performed with an analyzer TruSpec CN (LECO, 2021). The

equipment was calibrated with grains with high and low N_G certified by Leco Company.

Table 1. Characterization of the experimental sites. OM: organic matter. Bray P: Bray extractable phosphorus. NO3-- N: available N content at a 0 -60 cm depth at sowing. PD: planting date. HO: high oleic. C: conventional.
Tabla 1. Caracterización de los sitios experimentales. OM: materia orgánica del suelo. P Bray: fósforo extractable. N- NO3-: contenido de N disponible a la siembra de 0-60 cm de profundidad. PD: fecha de siembra. HO: alto oleico. C: convencional.

Site	Location	PD	Population	Genotype	Hybrid	Antecesor	OM	pH	Bray P	NO_3- N
			pl ha^{-1}				g kg^{-1}		mg kg^{-1}	kg ha^{-1}
1	38° 14' 20.77" S-57° 72'78.79" W	15-oct	62.000	C	Paraiso 1000 CL plus	Soybean	62	6.0	13.1	106.0
2	38° 14' 20.77" S-57° 72'78.79" W	15-oct	62.000	C	Paraiso 1000 CL plus	Soybean	53	6.5	12.2	115.0
3	37° 69' 20.56" S-58° 41'65.52" W	15-oct	60.000	C	Paraiso 102 CL	Soybean	52	5.9	5.9	73.0
4	37° 05' 20.60" S-57° 23'35.60" W	23-sep	60.000	HO	NTO 1.0 CL	Soybean	61	5.6	8.8	76.6
5	37° 05' 33.85" S-57° 25' 56.87" W	29-sep	60.000	HO	NTO 1.0 CL	Corn	57	5.6	6.8	69.7
6	37° 06' 03.10" S-57° 25' 53.19" W	22-sep	40.000	HO	SYN 3960 CL	Soybean	58	5.7	9.2	119.6
7	38° 05' 18.57" S-58° 13' 25.24" W	19-oct	66.000	HO	NTO 1.0 CL	Corn	32	6.4	7.0	54.1
8	38° 12' 10.33" S-57° 56' 16.68" W	18-oct	50.000	HO	NTO 1.0 CL	Soybean	67	5.9	16.5	75.5
9	38° 12' 44.66" S-57° 57' 04.02" W	13-oct	60.000	C	SYN 3970 CL	Corn	67	5.8	17.2	39.5
10	38° 30' 37.59" S-58° 48' 31.38" W	15-oct	62.000	C	Paraiso 102 CL	Soybean	51	6.0	15.1	43.0
11	38° 33' 13.80" S-58° 50' 36.39" W	17-oct	60.000	C	ADV CF 201	Soybean	44	6.1	13.7	44.5
12	38° 35' 17.60" S-59° 08' 27.00" W	16-oct	65.000	C	Paraiso 104 CL	Corn	57	6.0	13.1	44.1
13	38° 30' 34.07" S-60° 05' 42.10" W	14-oct	55.000	HO	ADV 5203 CL	Soybean	36	6.1	10.0	91.1
14	38° 40' 46.87" S-60° 08' 35.98" W	21-oct	55.000	HO	Aromo 105 CL	Soybean	45	6.1	13.2	70.3

Determination of N_G with NIRS technology

The samples were measured in a Near Infrared Spectroscopy Equipment (InlabNIR TEC-NIR-256, Tecnocientífica). Milled samples were stabilized at room temperature, and individually placed in the equipment capsule. The conditions of the equipment were adjusted to read at least 115 spectrums of each sample to ensure the measurement representativeness. Each measurement took less than 60 sec. Those spectrums were average to obtain the N concentration of each sample. The NIRS equipment was previously calibrated using samples measured with the Dumas method as reference. The calibration method was carried out with 208 samples covering a N concentration range from 1.5 to 3.9% N_G, and it was validated with 105 independent samples. The standard error of the prediction was 0.2%.

Grain oil extraction and determination of N in the meal

Twenty-two (22) samples were selected from the 84 sunflower grain samples, covering a wide range of N_G (1.75-3.80%) according to the values obtained with the three methodologies. The samples were milled and the oil was extracted by using n-hexane (Soxhlet) at 80°C during 8 h. The defatted samples were dried at 60ºC until constant weight to eliminate the rest of solvent and N_G was determined with the Dumas method. To calculate P_M of the defatted samples, we used a factor of 5.3 (Jones, 1941), and considered the percentage of residual hull of 10% according to Peyronnet *et al*. (2012).

Data analysis

Precision (dispersion parameter) and accuracy (position parameter) were

determined for each method. The precision is the proximity among results of independent determinations. It can be accessed via the repeatability, using the same procedure in a single sample, with the same operator, in short periods, using the same equipment in the same laboratory (OAA, 2008). This repeatability of the methods was assessed with the standard deviation (SD) and the variation coefficient (CV). The accuracy indicates the proximity between the average of measurements and the real value (OAA, 2008). It was evaluated with the bias (difference between the observed value and the reference value), and the percentage of relative difference (DRP) (INTI, 2021).

To determine the precision and accuracy of the methods, two sunflower grain standards with different N_G were used (Leco Corp. N_G-high = 3.36% and N_G-low = 1.72%). The standards were measured 30 times with each methodology and the mean value, SD, CV, bias and DRP were calculated. The mean values of N_G quantified with Kjeldahl, Dumas and NIRS were assessed with an ANOVA analysis with the procedure PROC GLM (R Core Team, 2021) and the Least Significant Difference (LSD, 5%).

Correlation and linear regression analysis between the methods were performed with all samples (n=84), and the intercept and slope of the regressions were compared with the 1:1 line with the Dummy method (5%). In addition, the absolute differences of the N_G measurements with the three methods were plotted and compared with the concordance curve of Dumas-Kjeldahl and Dumas-NIRS using the test PetoWilcoxon (Raggio *et al.*, 2003).

The relationship between P_M and N_G for Dumas, NIRS and Kjeldahl was described with lineal models. The slope and intercept of the three models were compared to asses if a unique equation could be developed to explain the relationship between P_M and N_G.

RESULTS AND DISCUSSION

Yield, oil and protein

Mean sunflower yield of the experiment was 3476 kg ha^{-1} (from 1804 to 5406 kg ha^{-1}). These results are in accordance with those reported by ASAGIR (2021) for the southeastern Buenos Aires Province (mean yield 3692 kg ha^{-1}) and by Zamora & Massigoge (2008) for south-central and western regions of the province (mean yield 3000-3200 kg ha^{-1}). The average of O_G was 50.7% (from 42.7 to 59.2%). These oil concentrations are higher than those reported for the Argentine National Trial Network of Commercial Sunflower Hybrids during the growing season 2016-2017 (43% to 53% ± 10%) (ASAGIR, 2021); probably because of the adequate hydric conditions of our experiments. Regardless of the methodology, N_G ranged from 1.57 to 3.80% indicating that a wide range of N concentration was obtained (**Figure 1**). The N_G of 79% of the samples (n= 66) were in

the range 2-3% values in accordance with those reported by Echeverría (2008) for southeastern Buenos Aires Province (2.1 to 3.1%). Only 11% and 10% of the samples presented N_G values lower than 2% or higher than 3%, respectively (**Figure 1**).

Precision and accuracy of each methodology

The estimation of N of the standard samples (Leco Corp. N_G-high = 3.36% and N_G-low = 1.72%), showed differences among methods ($p < 0.05$). These differences among methods could be attributed to the fact that each one determines different forms of N. Kjeldahl method only quantifies organic N, which passes into the ammonia form and is quantified as ammonium, whereas Dumas and NIRS methods also quantify other N forms. Dumas determines total N, including inorganic fractions as nitrite and nitrate (Daun & DeClercq, 1994; Simonne *et al.,* 1997), and NIRS measures the light absorption by the sample due to bonds or protein bands (Wells, 2006). However, Dumas and Kjeldahl presented similar values for the N estimation of the standard N_G-high, while both differed with NIRS (**Table 2**). Lanza *et al*. (2016) estimated total N for different types of meals and observed no differences in the estimation between Kjeldahl and Dumas. However, from the practical point of view, light differences were observed between Dumas and NIRS for N_G-low (0.27%) and Kjeldahl and NIRS for N_G-high (0.17%). These differences represent less than 1.5% P_G, indicating that any of the tested methodologies could be satisfactorily used to quantify N_G.

The NIRS method was the most precise (considering SD and CV), meaning that it had good repeatability in N_G estimations. The second method was Dumas and the last one was Kjeldahl, which presented the highest CV values (**Table 2**). Unlike the results observed in this work, Mihaljev *et al*. (2015) reported that NIRS presented high relative standard deviation for estimations of N in meat and else, lower precision than Dumas and Kjeldahl which showed high precision and narrow variation. On the other hand, Blanco & Villarroya (2002) reported that the estimations with NIRS presented similar precision than other analytical methods because the sample is barely manipulated. Lanza *et al*. (2016) reported that the Kjeldahl method produced high deviations in the estimation of total N of food samples compared to those obtained with Dumas. According to these authors, the Kjeldahl method involves a high number of steps and factors where the error of the operator and the pre-treatment of the samples may result in a high dispersion of the results. However, in our evaluation, the CV for Kjeldahl was acceptable in all cases (< 5%).

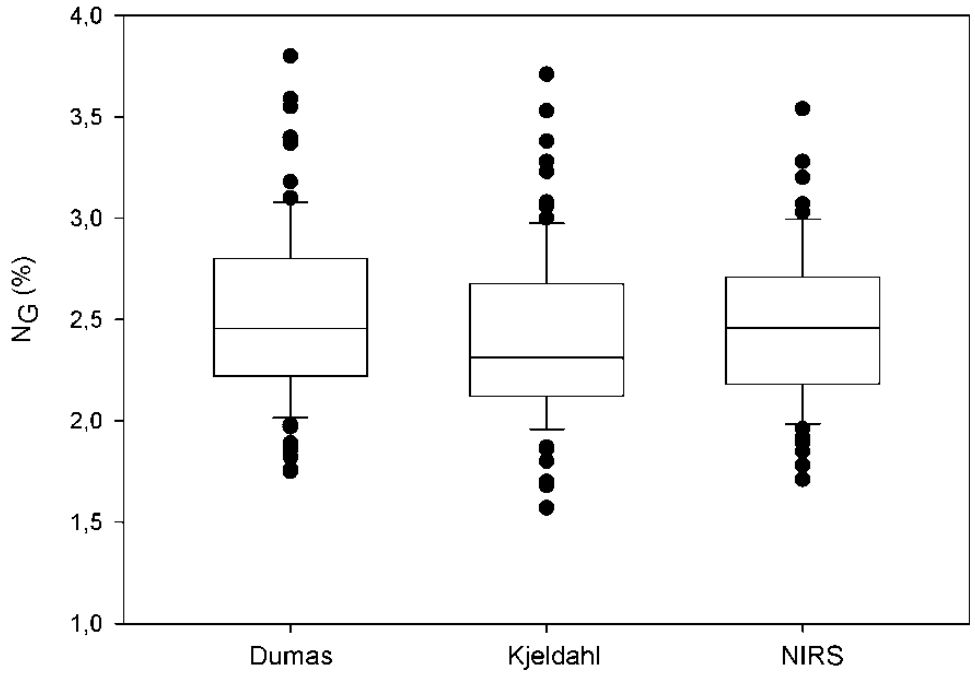

Figure 1. Grain Total nitrogen concentration (NG) by Dumas, Kjeldahl and NIRS methods in sunflower. Box lines are percentile 0.25, 0.50 and 0.75; whiskers are 0.10 and 0.90, and dots are 0.05 and 0.95 percentile (n= 84).
Figura 1. Concentración de nitrógeno total en grano (NG) de girasol para Dumas, Kjeldahl y NIRS. Las líneas de cada caja son percentiles 0.25, 0.50 y 0.75; los bigotes son 0.10 y 0.90, y los puntos son percentiles 0.05 y 0.95 (n = 84).

Table 2. Average (%), standard deviation (SD), coefficient of variation (CV) (%), bias (%) and percentage relative difference (PRD) for two Leco reference standards of sunflower grain with different concentration of total nitrogen (NG-high and NG- low), determined by the Dumas, Kjeldahl and NIRS methods.
Tabla 2. Promedio (%), desvío estándar (SD), coeficiente de variación (CV) (%), sesgo y diferencia relativa porcentual (PRD) para dos estándares de referencia Leco, en grano de girasol con diferente concentración de nitrógeno total (NG-alto and NG- bajo), determinado por los métodos Dumas, Kjeldahl y NIRS.

Standards	Method	Average (%)	SD	CV (%)	Bias (%)	PRD
$N_{G\text{-high}}$	Dumas	3.41*a	0.06	1.64	0.05	1.55
	Kjeldahl	3.43*a	0.17	4.87	0.07	2.16
	NIRS	3.26*b	0.01	0.20	- 0.10	- 3.01
$N_{G\text{-low}}$	Dumas	1.69*c	0.03	1.59	- 0.03	- 1.88
	Kjeldahl	1.75*b	0.08	4.71	0.03	1.60
	NIRS	1.96*a	0.02	1.10	0.24	13.68

* Reference value of the Leco Corp. NG-high standard = 3.36% and NG-low = 1.72%.
Different letters within columns indicate significant differences between Standard Value of NG as determined by the LSD test ($p < 0.05$).
* Valor de referencia de Leco Corp. Estándar NG-alto = 3.36% y NG-bajo = 1.72%.
Las diferentes letras dentro de las columnas indican diferencias significativas entre el Valor estándar de NG según lo determinado por la prueba LSD ($p < 0.05$).

Although the NIRS method was the most precise, using both standards, the most accurate methods were Dumas and Kjeldahl, which presented lower bias and DRP than NIRS (**Table 2**). The NIRS tended to overestimate the N concentration of the standard N_G-low and to underestimate N_G-high value, probably because NIRS is an

indirect method and its accuracy depends on the reference method used for calibration (Sáez-Plaza *et al.*, 2013)

Comparison of the three methods

Figures 2a, **2b**, and **2c** show significant relationships between Dumas, Kjedahl and NIRS methods in determining N_G. In all cases, the coefficients of determinations were higher than 0.80 and the correlation was statistically significant ($p < 0.001$) between the different methods. According to these results, the three methods could be used to determine N_G. Esquivel-Valenzuela *et al*. (2018) reported that NIRS is an alternative to the traditional methods of analysis, with potential to obtain values of soil properties (e.g. organic carbon, total N) fast and with precision. Watson & Galliher (2001) reported a narrow correlation (r=0.96) between the estimations of N_G of farm products (seeds, plant tissues, manure, water, etc.) with Kjeldahl and Dumas methods. Other authors reported that Dumas and Kjeldahl methods showed similar results for N_G of soil and vegetable products (Simonne *et al.*, 1997). In this study, the relationship between the N_G content determined by NIRS and Dumas showed the highest degree of association ($r^2 = 0.93$) (**Figure 2a**), and highest correlation coefficient ($r = 0.96$). The correlation between the other methods was slightly lower ($r = 0.91$ for Kjeldahl-Dumas, and 0.90 for NIRS-Kjeldahl).

Nevertheless, in the three relationships, the slopes between observed vs predicted data differed from 1 ($p < 0.001$); and the intercepts were different from 0 ($p < 0.001$). At high N_G concentrations, the methods NIRS and Kjeldahl sub estimated N_G compared to the Dumas method (Figure 2a and 2b, respectively). However, at low N_G concentrations, NIRS tended to overestimate N_G whereas the Kjeldahl method was similar to Dumas (**Figure 2a** and **2b**). Jung *et al*. (2003) reported that the N_G values of soybean by-products determined by Kjeldahl were slightly lower than those obtained with Dumas. Similar results were obtained by Daun & DeClercq (1994) in oleaginous grains (canola, soybean and sunflower), attributing that differences among methods to the inability to use

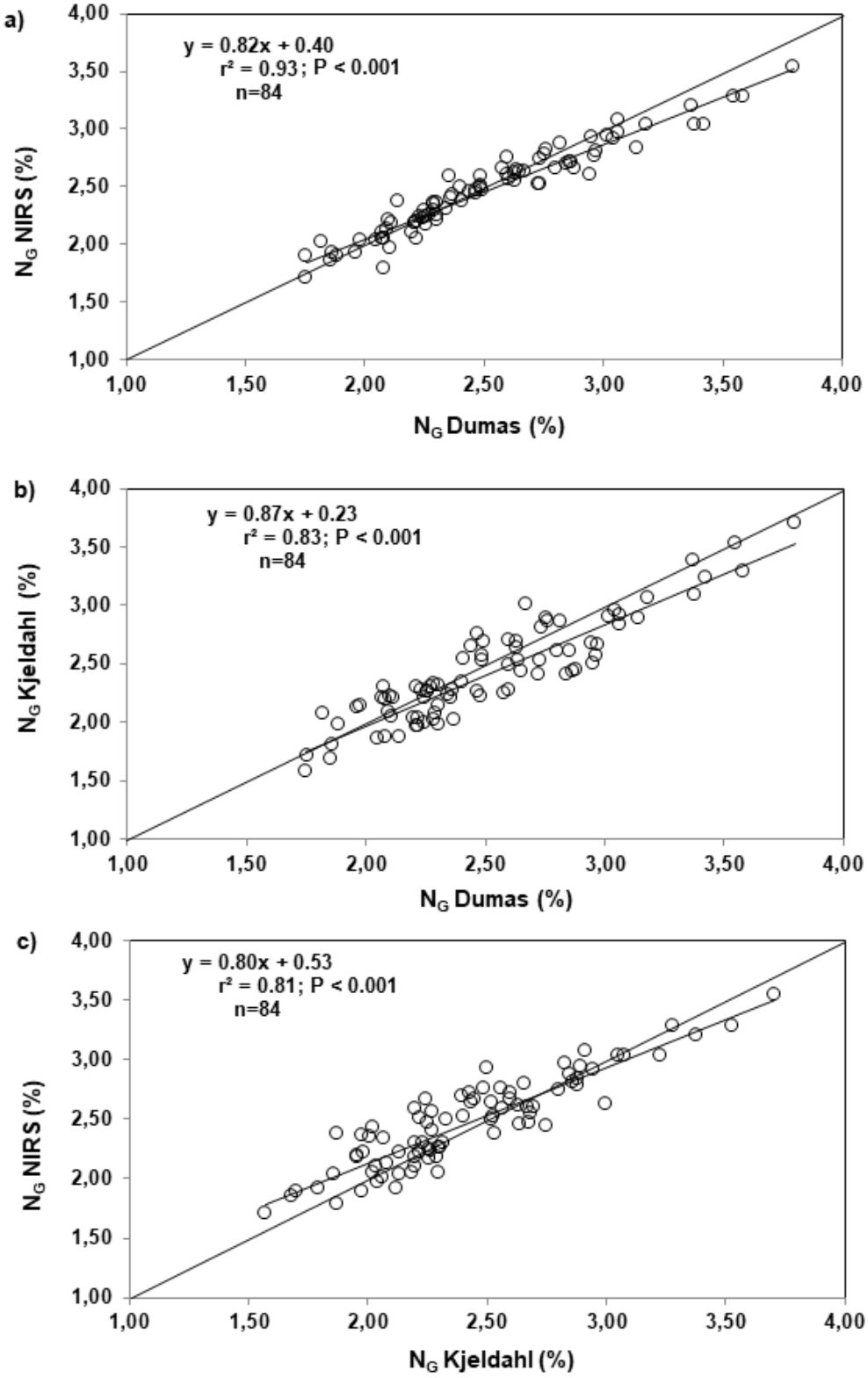

Figure 2. Relationship between grain total nitrogen concentration (NG) in sunflower determined by a) NIRS and b) Kjeldahl with respect to Dumas and, c) NIRS with respect to Kjeldahl. Dotted line equals the 1: 1 line. n = number of cases.
Figura 2. Relación entre la concentración de nitrógeno total en grano (NG) de girasol determinada por a) NIRS y b) Kjeldahl con respecto a Dumas y, c) NIRS con respecto a Kjeldahl. La línea punteada es igual a la línea 1: 1. n = número de casos.

mercury as catalyzer in the Kjeldahl method. Watson & Galliher (2001) observed that the correlation between Kjeldahl and Dumas method improved to 0.99 when the samples with high nitrate concentrations were excluded. Simonne *et al*. (1997) observed that in products with high nitrate concentration, the Dumas method showed higher values of N because this method quantifies inorganic N form not quantified by Kjeldahl. So, for N_G concentrations of sunflower grains lower than 3% approximately (**Figure 2a** and **2b**), and considering that the most usual N concentrations in these grains are between 2.1 and 3.1% (Echeverría, 2008), NIRS or Kjeldahl methods could be a trustworthy alternative as they predict similarly to Dumas method.

On the other hand, when comparing N_G determined by NIRS with respect to Kjeldahl, NIRS over- and under-estimated N_G at low and high N concentrations, respectively (**Figure 2c**). Similar results were observed with NIRS compared to Dumas method (**Figure 2a**). Acuña (2005), in a study predicting the nutritional composition of foods by NIRS, pointed out that the high levels of error were a consequence of a weak relationship between the NIRS prediction and the reference values, since the data were overestimated and underestimated by the NIRS technique. Vivar (2009) comparing the protein values of hydrolyzed feather meal determined by NIRS with respect to Kjeldahl observed that the NIRS tended to underestimate and overestimate these values. Other researchers reported that this behavior could be explained because NIRS and Kjeldahl quantify different characteristics.

The concordance of the NIRS and Kjeldahl methods with Dumas from absolute differences is shown in **Figure 3**. NIRS presented higher agreement level with Dumas than Kjeldahl ($p< 0.05$). Considering a tolerance limit of 0.20%, the agreement between Dumas-NIRS and Dumas-Kjeldahl was of 90% and 60%, respectively (**Figure 3**). Moreover, NIRS presented a better perform respect Kjeldahl for the whole range of comparison. Therefore, NIRS could be a reliable alternative to Dumas, given the high concordance with this reference method in the determination of N_G respect to Kjeldahl. In addition, NIRS has the advantage of being a low-cost method compared to Dumas (Velasco et al., 2004).
The NIRS method, presents greater accuracy, does not use chemical products, does not generates toxic waste, demands little time to analysis, it is simple and it is a non-destructive or invasive technique since it requires a minimum preparation of the sample (Blanco & Villarroya, 2002; Bosco, 2010). In addition, NIRS allowed the determination of oil concentration and fatty acid composition simultaneously with N_G, representing an additional advantage over the other methodologies (Blanco & Villarroya, 2002; Velasco *et al.*, 2004).

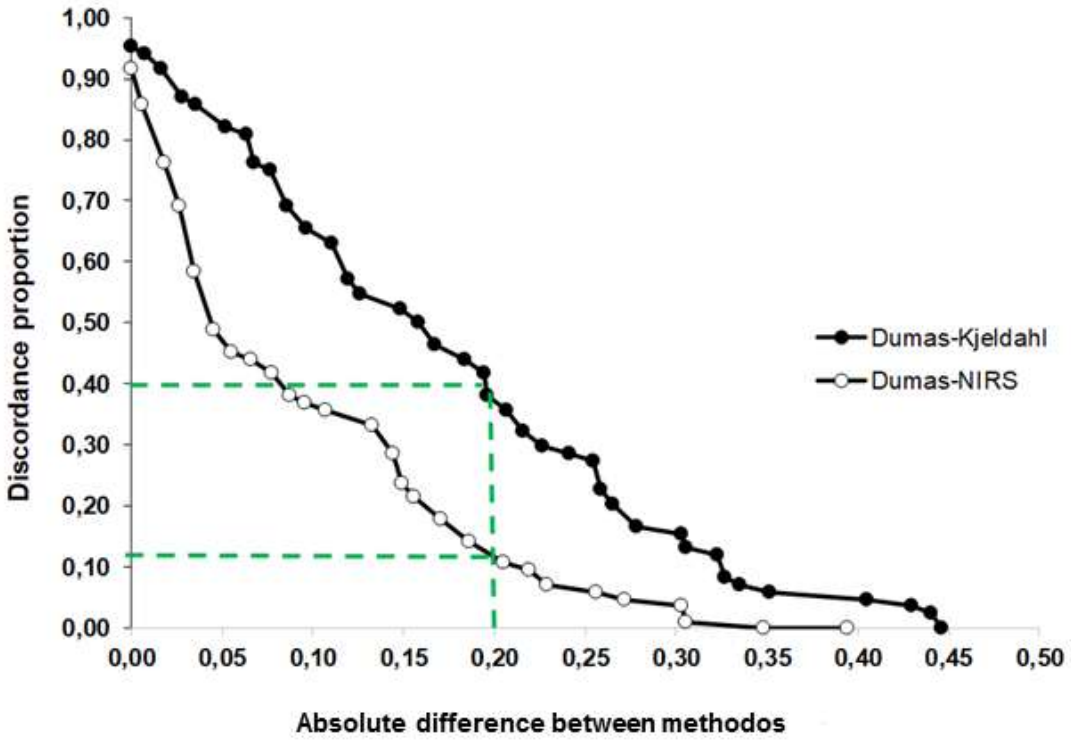

Figure 3. Discordant proportion of observations between Dumas-NIRS and Dumas-Kjeldahl. The dotted line indicates the proportion of discordance for an absolute difference between methods of 0.20.
Figura 3. Proporción de discordancia de observaciones entre Dumas-NIRS y Dumas-Kjeldahl. La línea punteada indica la proporción de discordancia para una diferencia absoluta entre los métodos de 0,20.

Estimation of P_M

The P_M under the dehulling conditions, as described in materials and methods section, was estimated from N_G concentrations determined by each of the methodologies (Figure 4). The N_G explained 59%, 67%, and 57% of the variation in P_M values for Dumas, NIRS and Kjeldahl, respectively (**Figure 4 a**, **b** and **c**).

In addition, comparing the three models, the slopes and the intercepts were similar, thus a single model explained the relationship between P_M and N_G (r^2 = 0.60) (**Figure 4 d**). For P_M estimations, it is important to define the dehulling and oil extraction conditions, as the relationship between N_G and P_M will vary according to the oil and hull remains in the meal. The NIRS could be a better alternative to the other methods, mainly because it is fast and inexpensive. This would be of great importance within the market for the commercialization of by-products since the estimation of P_M would indicate the quality of pellets. Grains with 2.1% of N_G would result in by-products with 36% protein, and else, differential prices in the market (de Figueiredo *et al.*, 2015; González-Pérez, 2015; Dauguet *et al.*, 2016). Moreover, although any of the three methods could be used to determine N_G and estimate P_M, the

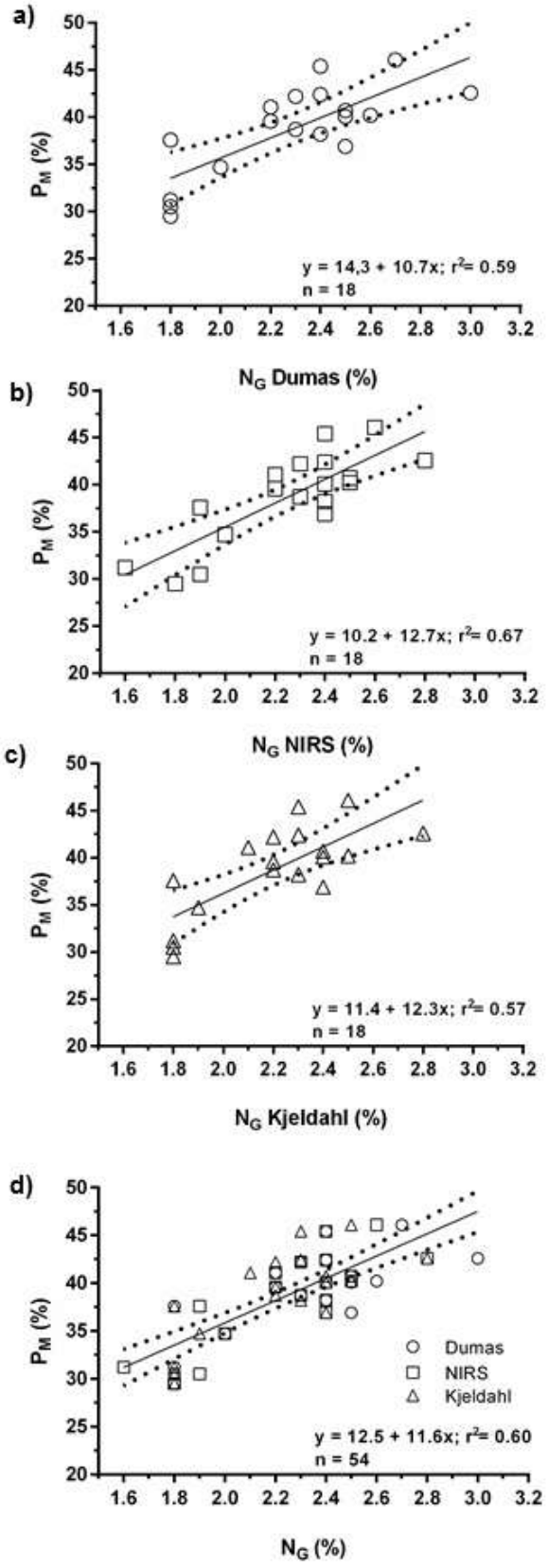

Figure 4. Relationship between the percentage of protein in byproducts (PM) and total grain nitrogen (NG) in sunflower determined by a) Dumas, b) NIRS, c) Kjeldahl and d) the three methodologies (global model). n = number of cases. Dotted lines indicate the 95% confidence interval.
Figura 4. Relación entre el porcentaje de proteína en subproductos (PM) y el nitrógeno total de grano (NG) de girasol determinado por a) Dumas, b) NIRS, c) Kjeldahl y d) las tres metodologías (modelo global). n = número de casos. Las líneas punteadas indican el intervalo de confianza del 95%.

NIRS has the advantage of being a fast, economic, non-toxic, accurate and a precise method.

CONCLUSIONS

NIRS was the most precise method for the determination of N_G, while Dumas and Kjeldahl were the most accurate methods. In addition, NIRS presented a greater degree of agreement with Dumas than Kjeldahl.

The P_M value might be estimated from N_G independently of the methodology. Although further studies are necessary to validate the proposed methods and models, they represent a significant advance to estimate P_M, a character that determines the quality of by-products of the oil industry.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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