JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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A Simple Analytical Method for Dhurrin Content Evaluation in Cyanogenic Plants for Their Utilization in Fodder and Biofumigation

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ABSTRACT: Cyanogenic plants have some potential as biocidal green manure crops in limiting several soilborne pests and pathogens. Sorghum (*Sorghum bicolor* (L.) Moench) and Sudangrass (*Sorghum bicolor* subsp. *sudanense* (P.) Stapf), in fact, contain the cyanogenic glucoside *p*-hydroxy-(*S*)-mandelonitrile-β-D-glucoside (dhurrin) as a substrate of its secondary defensive system able to release hydrogen cyanide following tissue lesions due to biotic or abiotic factors. Given that dhurrin content is correlated with the biofumigant efficacy of the plants, a high dhurrin content could be a positive character for utilization of sorghum and Sudangrass as biocidal green manure plants. For chemical characterization of the available germplasm, a simple, safe, and accurate method is necessary. In this paper, a new method for dhurrin analysis, based on methanol extraction and high-performance liquid chromatography, is reported and discussed. The feasibility of this analytical procedure was tested by evaluating dhurrin level in roots and stems during cultivation of four different sorghum and Sudangrass varieties in agronomic trials performed in 2008 in the Po valley (Italy). The dhurrin content ranged from 0.16 ± 0.04 to 7.14 ± 0.32 mg g^{-1} on dried matter (DM) in stems and from 1.38 ± 0.02 to 6.57 ± 0.09 mg g^{-1} on DM in roots, showing statistical differences among the tested germplasms that could be linked to the efficacy of their utilization as biofumigant plants. The method also opens new perspectives for the characterization of sorgum plants as fodder, for which the presence of dhurrin is considered to be negative for its well-known toxicity.

KEYWORDS: biofumigation, dhurrin, sorghum, Sudangrass, hydrogen cyanide, cyanogenic glycosides, *p*-hydroxybenzaldehyde

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) and Sudangrass (*Sorghum bicolor* subsp. *sudanense* (P.) Stapf) are heat- and drought-tolerant cover crops with interesting potential for improving soil quality, suppressing weeds, and controlling diseases and nematode damage.[1,2] The cells of Sudangrass and sorghum contain the cyanogenic glucoside *p*-hydroxy-(*S*)-mandelonitrile-β-D-glucoside (dhurrin) which can degrade into hydrogen cyanide (HCN) in a process known as cyanogenesis. Figure 1 shows the two-step enzymatic hydrolysis process that gives rise to the release of hydrogen cyanide. Dhurrin is first hydrolyzed by the endogenous β-d-glucoside glucohydrolase (dhurrinase) (EC 3.2.1.21) to produce glucose and *p*-hydroxy-(*S*)-mandelonitrile. This latter compound is unstable and quickly converted to free HCN and *p*-hydroxybenzaldehyde (*p*-HB) by the endogenous enzyme α-hydroxynitrile lyase or at basic pH values.[3] HCN is a powerful nematicidal compound[4−8] that plays a role in the low sensitivity of these plants to several pests and pathogens. As observed for the glucosinolate−myrosinase system in Brassicaceae plants,[9] in intact plant tissues enzymes and substrate are kept separated in the cells: dhurrin is located in the vacuole of the epidermal cells, whereas the catabolitic enzymes are in the mesophyll cells.[3] Only when plant tissues are lesioned or destroyed, as a consequence of biotic or abiotic factors, do enzymes and substrates come into contact, releasing the bioactive compound that is involved in limiting plant infection.

Due to the negative role of cyanogenic glucosides in human nutrition and fodder, various methods, including direct high-performance liquid chromatography (HPLC)[10,11] and indirect evaluation of released HCN after hydrolysis,[12] have been used for evaluating these compounds in plants. For example, HCN liberated from plant material can be assessed by colorimetric methods such as the succinimide/barbituric method[13] and the alkaline picrate method.[14] However, all of the reported indirect methods are described as time-consuming and are not practical for screening a large number of samples without the use of a hazardous cyanide salt for calibration curve determination, whereas in most cases the direct HPLC methods require the use of an expensive cyanogenic glucoside standard that is, in addition, not always easily available on the market.

With the aim of characterizing a large number of genotypes and tissues of Sudangrass and sorghum−Sudangrass hybrid plants (viz., from agronomic trials or breeding trials) a simple and accurate direct analytical method has been defined. The proposed method is also cheap and safe and allows a quantitative and fast determination of dhurrin in plants. It can be easily performed for routine analysis by means of the use of an HPLC instrument equipped with an automatic injector. This new method was applied in the evaluation of dhurrin content on different varieties of sorghum and Sudangrass during cultivation in agronomic trials performed in Italy in 2008, confirming its feasibility and practicality.

MATERIAL AND METHODS

Reagents. Dhurrin standard (CAS Registry No. 499-20-7) was obtained from Extrasynthése (Lyon, France) and *p*-hydroxybenzaldehyde (CAS Registry No. 123-08-0) from Sigma-Aldrich Chemie (Steinheim, Germany). Activated carbon used in this study was charcoal,

Received: February 25, 2011
Revised: June 23, 2011
Accepted: June 27, 2011
Published: June 27, 2011

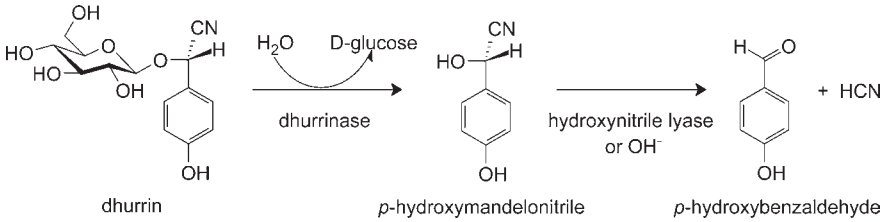

Figure 1. General pathway of the enzymatic hydrolysis of dhurrin cyanogenesis.

decolorizing powder activated, acid washed, and purchased from BDH Ltd. (Poole, U.K.). Acetonitrile was of HPLC grade, whereas other chemicals were of analytical grade.

Plant Materials. Cultivars in our investigations included two sorghum (cv. Piper and Super Dolce 10) and two Sudangrass (cv. Trudan 8 and Sordan 79). The seeds were obtained on the seed market, where they are commercialized as biocidal green manure (Trudan 8 and Sordan 79) or forage crops (Piper and Super Dolce 10).

Plant Cultivation. In 2008, a sorghum and Sudangrass plant cultivation trial was carried out in the environment of Budrio (Bologna, Italy) located in the Po valley (Italy; latitude 44° 32′ 13″ N, longitude 11° 29′ 40″ E, altitude 29 m asl), in plots (15 m^2) arranged in a randomized block design, with three replicates. The soil was medium clayey with a good phosphorus and potassium content and was amended with 60 units of nitrogen. The four cultivars were sown in the first week of May, with a seed density of approximately 5 g m^{-2}, distributed in continuous rows with an inter-row spacing of 0.15 m. No irrigation was required during the entire cultivation cycle, apart from an irrigation treatment after sowing to anticipate seed germination, and no pesticide treatment was, as expected, necessary.

Sample Preparation and Extraction. Every week from sprouting to flowering time, three plant samples of hypogeal and epigeal parts of each selection were taken and weighed. One sample was used to evaluate the dry matter (DM) content by oven-drying it at 105 °C overnight. The other two samples were weighed and immediately frozen at −20 °C. Plant samples were then freeze-dried using a Minifast D0.1 freeze-drier (Edwards High Vacuum Milan, Italy) (from −40 to +18 °C in 2 days, with a reduced pressure of 0.1 mbar). Freeze-dried materials were ground to a fine powder in a mill. For the analysis, 0.2 g of plant tissue sample was weighed into a 25 mL centrifuge tube and 0.1 g of activated carbon was added. Hypogeal plant tissue samples were treated in the same way, with the only difference being that the amounts were 0.1 and 0.05 g for sample and activated carbon, respectively. After the addition of 10 mL of MeOH, the tube was exposed at room temperature to ultrasound (40 Hz) for 25 min in a Sonica Sweep System model 4200EP ultrasonic water bath (Soltec, Milan, Italy). The mixture was then left overnight in the tube and centrifuged with a J2-MC centrifuge (Beckman, Palo Alto, CA) at 17000*g* for 30 min at 10 °C the day after. The supernatant solution was filtered through a Whatman no. 4 filter paper, and water 1:1 (v/v) was added to the resulting clear solution. One milliliter of the diluted solution was withdrawn, transferred into an autosampler vial, and analyzed by reverse phase HPLC.

HPLC Analysis. Dhurrin was analyzed according to a modified HPLC procedure previously developed by Johansen et al. for pure dhurrin.[15] The analyses were carried out using an HPLC model 1100 (Agilent Technologies, Waldbronn, Germany) equipped with an Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm particle size) thermostated at 35 °C, an automatic injector, and a diode array as detector. The chromatography was performed with 1 mL min^{-1} flow rate by eluting with a gradient of water (A) and acetonitrile (B). The gradient program consisted of isocratic 10% B for 1 min, linear gradient to 30% B for 7 min, and linear gradient to 10% B in 2 min. Dhurrin was detected monitoring the absorbance at 232 nm. The peak corresponding to dhurrin was identified by comparing the retention time and spectra to that of pure dhurrin.

Dhurrin Quantification. For dhurrin quantification, internal and external standard methods were applied and compared.

External Standard (ES) Method. A stock water solution of pure dhurrin (1 mg mL^{-1}) was prepared and stored at −20 °C. Aliquots of the standard stock solution were properly diluted in H_2O/MeOH 1:1 (v/v) to obtain concentrations of 5, 10, 25, 50, and 100 mg L^{-1}. Twenty microliters of each calibration standard solution was injected 5-fold into the column, and peak areas were recorded. A dhurrin calibration curve was defined by plotting the peak area obtained with the five standard solutions against the corresponding known concentrations. The dhurrin stock solution was stored at −20 °C for 1 month, during which its stability was verified as already reported.[15] The stability of the dhurrin working standards was evaluated at every analysis session.

Internal Standard (IS) Method. As the internal standard, a stock water solution of *p*-HB (0.8 mg mL^{-1}) was prepared and stored at 4 °C. One hundred microliters of stock solution was added to 1 mL of the methanolic extract sample together with 1 mL of water. Twenty microliters of the resulting solution was then injected for HPLC analysis using the above-reported instrumental conditions.

Calculation of Dhurrin Concentrations (IS Method). The dhurrin concentrations of the samples were calculated by using the formula

$$C\ (\text{mg g}^{-1}) = (\text{area}_{\text{dhurrin}} \times 0.65 \times \text{MW} \times V_{\text{extr}})/(\text{area}_{p\text{-HB}} \times RF \times W_{\text{DM}} \times 1000)$$

where 0.65 refers to the quantity expressed in μmol of *p*-HB added in each analysis to 1 mL of sample extract and MW is the molecular weight of dhurrin (311.30 g mol^{-1}). V_{extr} is the extract total volume (mL) and W_{DM} (g) the sample weight. For dhurrin content evaluation the response factor (RF) relative to *p*-HB was considered. This was calculated as the ratio between the molar extinction coefficients (ε) at 232 nm in water for dhurrin relative to that of *p*-HB in the same experimental conditions.

Recovery Test. A recovery test was carried out, repeatedly analyzing an epigeal sample of Sudangrass cv. Piper characterized by a low dhurrin level (0.23 ± 0.02 mg g^{-1}). Five different freeze-dried finely ground plant tissue subsamples of this field sample were spiked with a defined amount of dhurrin standard solution. Pure dhurrin was dissolved in MeOH (5 mg mL^{-1}) and further diluted with MeOH to achieve the following concentrations: 10, 20, 50, 100, and 200 mg L^{-1}. One hundred microliters of each standard solution was added to 200 mg of ground plant tissue, extracted, and analyzed by HPLC as described before. Each sample was analyzed twice. The amount of dhurrin in the sample extracts was determined by using a previously defined calibration curve and compared with the added amount to determine the recovery.

Reproducibility of Dhurrin Analysis. The analysis method was checked for reproducibility in terms of both extraction and analysis. The reproducibility of the methanolic ultrasound extraction system was assessed by repeated extractions performed on five subsamples of the same ground plant epigeal tissue Super Dolce 10 as described above. Each sample was analyzed 5-fold to determine HPLC analysis repeatability.

p-HB Molar Extinction Coefficient Determination. The molar extinction coefficient (ε) of *p*-HB was determined by using a double-beam Cary model 219 UV−vis recording spectrophotometer (Varian, Palo Alto, CA) equipped with 1 cm quartz cells. A stock water solution of *p*-HB (0.8 mg mL^{-1}) was prepared, and 0.5, 1, and 1.5 mL aliquots were diluted in 10 mL volumetric flasks with water to achieve final concentrations of 0.13, 0.33, and 0.46 mM, respectively. The absorbance of each final solution was measured at 232 nm in triplicate against a water blank. Absorbance mean values were plotted against the corresponding molar concentrations, and the ε was calculated by linear regression.

Statistical Evaluation. Data are expressed as the mean ± SD; recovery and reproducibility are reported with RSD. The dhurrin calibration curve equation was evaluated by fitting plot data of area versus concentration by linear regression. The computer program used for all linear regressions was SigmaPlot 9.0.1 (Systat Software Inc.). The results of dhurrin quantification in plant samples were statistically analyzed using completely randomized design ANOVA performed with SigmaStat 3.11 (Systat Software Inc.). When ANOVA showed statistical differences ($P \leq 0.01$ or ≤ 0.05), LSD Fisher's protected test ($P \leq 0.05$) was applied for mean separation. Moreover, statistical significance (ANOVA) of linear regression between analytical methods was tested with the same statistical software. Finally, Pearson's correlation test (r) was applied to evaluate the degree of relationship between analytical methods to validate the linear model.

RESULTS AND DISCUSSION

Dhurrin Calibration Curve. For quantitative analysis purpose, a five-point dhurrin calibration curve in the range of 5−100 mg L^{-1} was obtained. Linear regression analysis of the peak area response (y) versus the concentration value expressed as milligrams per milliliter (x) gave the following equation: $y = 4 + 38802x$. The correlation coefficient value ($r^2 = 0.9999$) demonstrated a very high linearity of the method over the explored concentration range.

Internal Standard Method. To avoid the use of dhurrin standard and to make the analysis easier and cheaper, it was decided to find a suitable alternative IS to add to the extract sample before HPLC determination. *p*-HB was chosen as the internal standard due to its low cost and widespread availability. Under the defined chromatographic conditions, it did not interfere with any other peaks present in the analyzed samples. The retention times (t_R) of dhurrin and *p*-HB were about 4.28 and 7.35 min, respectively, as shown in Figure 2 for a field sample of Sordan 79.

For dhurrin content evaluation, an RF value of 2.78 relative to *p*-HB was determined. For dhurrin, the ε (232 nm, water) value of 1.05×10^4 M cm^{-1} was used as reported by Nahrstedt et al.[16] for this cyanogenic glucoside, whereas for *p*-HB a molar extinction coefficient of 3.77×10^3 M cm^{-1} was evaluated spectrophotometrically in the same experimental conditions.

Comparison of Results Obtained by Using Internal and External HPLC Analysis Methods. The two above-described methods were compared using 19 freeze-dried, finely ground epigeal plant tissue samples of the four different varieties cultivated in the Po valley in 2008. The samples were chosen to cover the range of dhurrin content present in the different varieties (from 0.16 ± 0.04 to 7.14 ± 0.32 mg g^{-1} as determined by using the IS method). Results obtained by the two methods proved that they were highly correlated to each other. Figure 3 shows the linear regression of the data obtained with the two compared HPLC methods calculated

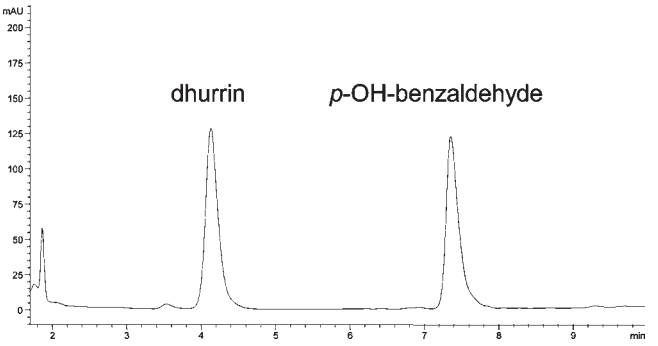

Figure 2. HPLC chromatogram showing the analysis of a real epigeal extract sample of Sordan 8. This clearly shows the good separation between dhurrin and *p*-hydroxybenzaldehyde (IS) peaks.

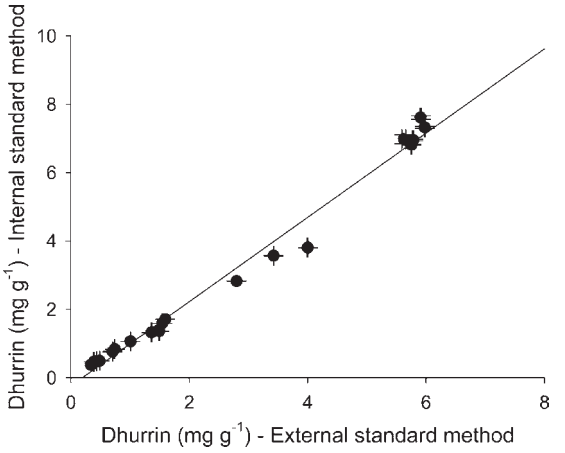

Figure 3. Correlation between external and internal standard analytical HPLC methods for dhurrin quantification in sorghum and Sudangrass.

by using the equation

$$dhurrin\ (mg\ g^{-1})_{IS} = -0.24 + 1.23\ dhurrin\ (mg\ g^{-1})_{ES}$$

with a fit of $r^2 = 0.9867$ that confirmed the good correlation between these two methods. The IS method is preferable to the external one for two main reasons: the internal method offers the advantage of deleting every instrumental fluctuation given that the added standard is always analyzed at the same time and under the same instrumental conditions as the analyte of interest, whereas the performances of the second method have to be verified over time.

Recovery Efficiency Test. The sample extraction procedure was checked for evaluating recovery efficiency by spiked dhurrin standard, analyzed, and quantified by calibration curve. Recoveries were determined for the sample preparation procedure by spiking 0.1, 0.2, 0.5, 1.0, and 2.0 mg of pure dhurrin into 200 mg of a Piper Sudangrass epigeal sample characterized by a low dhurrin content. The amounts of the added standard were chosen to cover the range of dhurrin content present in the sample throughout the period of cultivation. Following the above-reported procedure, the recoveries provided values ranging from 87 to 98% as reported in Table 1.

Reproducibility of Dhurrin Extraction and Analysis by HPLC. Extraction of dhurrin was performed as described by Kobaisy

Table 1. Recovery of the New Extraction Method with Spiked Dhurrin Standard Added to 200 mg Subsamples of a Piper Epigeal Sample

sample	spiked dhurrin (mg)	recovery[a] (%)
1	0.1	98 ± 0.3
2	0.2	89 ± 0.0
3	0.5	87 ± 0.1
4	1.0	91 ± 0.0
5	2.0	88 ± 0.0

[a] Values are obtained by means of two repetitions comparing peak areas of spiked dhurrin to standard curve.

Table 2. Reproducibility of Dhurrin Extraction and HPLC Analysis by Means of Internal Standard Method[a]

sample	dhurrin (mg/g DM)	RSD[b] (%)
1	6.98 ± 0.13	1.9
2	6.81 ± 0.02	0.3
3	6.95 ± 0.03	0.4
4	7.61 ± 0.05	0.7
5	7.32 ± 0.03	0.4
mean	7.14 ± 0.32[c]	4.5[d]

[a] Determinations were performed on Super Dolce 10 subsamples. [b] Values were obtained by means of five injections with the internal standard method. [c] Mean ± standard deviation of five replicate extractions. [d] RSD of five replicate extractions.

et al.,[10] although modified by using pure MeOH instead of EtOH 70%, because this was found to better affect the process, confirming that MeOH is an excellent solvent for cyanogenic compounds as already reported for the extraction of amygdalin and prunasin in almond tree tissues.[17] Activated carbon was used to eliminate pigments, such as chlorophyll from epigeal plant samples and slightly yellow- to red-colored pigments present in roots. Activated carbon is a well-known decolorizing agent which, when added to the sample before MeOH extraction through sonication, made it possible to obtain clear transparent extracts showing no interfering peaks in HPLC chromatograms. After sonication, the samples were left at room temperature overnight before centrifugation and analysis, because this procedure gave a better extraction yield (data not shown). The extraction reproducibility was established on five different extracts of the same sorghum sample, analyzing each extract by 5-fold injections. Dhurrin was then quantified using the internal standard method with the addition of *p*-HB; the RSD % for replicated extractions was 4.5, and RSD % between injections varied between 0.4 and 1.9 as shown in Table 2.

Dhurrin Content in Sorghum and Sorghum Sudangrass Plant Materials. *Seeds.* The content of dhurrin in seeds of Piper, Trudan 8, Sordan 79, and Super Dolce 10 was lower than the limit of detectability of 0.10 mg g^{-1} as experimentally determined (data not shown). This finding was in accordance with data reported in the literature on different genotypes showing only traces or very low dhurrin levels in the seeds.[12,15,18]

Plants and Roots. Piper, Trudan 8, Sordan 79, and Super Dolce 10 were analyzed weekly for dhurrin content in both epigeal and hypogeal parts. Sampling was performed from the sprouting to flowering phases of the plants (Tables 3 and 4).

Table 3. Dhurrin in Epigeal Part of Sorghum (Super Dolce 10 and Piper) and Sudangrass (Trudan 8 and Sordan 79) Cultivars during Cultivation in 2008 (Determined by the IS Method)[a]

cultivar	June 10, 2008	June 17, 2008	June 23, 2008	July 1, 2008	July 8, 2008
Super Dolce 10	7.14 a	2.83 a	1.32 a	1.20 b	0.84 b
Sordan 79	2.58 b	1.45 b	1.85 a	1.84 a	1.03 a
Trudan 8	0.42 c	0.37 c	0.39 b	0.36 c	0.16 c
Piper	0.36 c	0.37 c	0.23 b	0.33 c	0.26 c
LSD (p = 0.05)	0.29	0.57	0.57	0.12	0.16

[a] Values are expressed as mg g^{-1} of dried matter. Different letters within the same column indicate a significant difference ($P \leq 0.05$).

Table 4. Dhurrin in Hypogeal Part of Sorghum (Super Dolce 10 and Piper) and Sudangrass (Trudan 8 and Sordan 79) Cultivars during Cultivation in 2008 (Determined by the IS Method)[a]

cultivar	June 10, 2008	June 17, 2008	June 23, 2008	July 1, 2008	July 8, 2008
Super Dolce 10	2.69	2.58 b	1.68 b	5.24 b	5.81 b
Sordan 79	3.02	2.68 b	1.63 b	6.57 a	4.98 c
Trudan 8	3.66	1.73 c	1.62 b	1.42 d	1.38 d
Piper	2.40	4.14 a	3.30 a	3.16 c	6.38 a
LSD (p = 0.05)	1.86	0.20	0.14	0.43	0.22

[a] Values are expressed as mg g^{-1} of dried matter. Different letters within the same column indicate a significant difference ($P \leq 0.05$).

Analyses of leaves, stems, and flowers of a Super Dolce 10 sample harvested at flowering phase gave dhurrin contents of 5.63 ± 0.19, 1.06 ± 0.03, and 1.57 ± 0.01 mg g^{-1}, respectively, confirming the variability in the different plant organs as already reported in the literature.[19]

The evaluation of dhurrin content of the whole epigeal part showed significant differences among the tested genotypes with Super Dolce 10 and Sordan 79 characterized by a higher content at every sampling time when compared to Piper and Trudan 8. This result confirms the applicability of green biomass in animal diet for these last two varieties, whereas it raises some doubts for Super Dolce and Sordan 79, considering the toxic effects derived from a higher level of dhurrin.[20,21] The four cultivars showed the same general trend with an inverse correlation between plant growth and the level of dhurrin in the epigeal part as reported in the literature.[22,23]

The hypogeal part evaluation, instead, showed how dhurrin content in root was, at almost all sampling times, significantly higher in Piper, followed by Super Dolce 10. Trudan 8, instead, showed the lowest dhurrin content also in roots (ranging around 1.6 mg g^{-1}), an aspect that could strongly discourage its application as a biocidal green manure plant.

Conclusions. This first experimental application of the reported new analytical procedure confirmed that its utilization is practical, cheap, simple, and suitable for routine analyses on a wide number of samples. The system could thus be applied in a wide and, moreover, safe way, in genetic improvement research studies for both fodder and biocidal applications. The evaluation of dhurrin content of new and old genotypes could be fundamental

for defining their field of application. For this purpose, the possibility of evaluating the dhurrin levels of different parts of the plant at different growing phases should also be considered.

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ABBREVIATIONS USED

DM, dried matter; *p*-HB, *p*-hydroxybenzaldehyde; HPLC, high-performance liquid chromatography; IS, internal standard; ES, external standard.

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