IDENTIFICATION AND MEASUREMENT OF BOTANICAL SPECIES IN ANIMAL FEED

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Abstract – Within the framework of the EU Project Feed-code, we developed and pre-validated a DNA-based protocol for the qualitative and quantitative determination of the botanical composition of dairy cow feed. Due to the biological complexity and intrinsic variability of the animal feed matrixes, we were confronted with several problematic issues. Hereby we present our current scheme and solutions, successfully tested on feed samples and raw materials

Keywords: feed composition, raw materials, reference mixtures, DNA calibrators, target copy number

1. INTRODUCTION

Identification and quantification of the different botanical species, declared or not, that are actually present in feed samples, although matter of important regulatory issues [1] are far from being effectively addressed. Currently, the only accepted method is based on the recognition of plant species done with the optical microscope [2], in the presence of a vast and worrisome contamination of circulating raw materials and the substantial absence of certified standards of reference. In addition, scientific articles addressing this issue are limited in number and proposed solutions [3, 4]. We took advantage of the special features of a new molecular marker we have developed (TBP; [5]) to address this problem with the financial support of the EC, granted by the Feed-code (http://www.feedcode-project.eu) project, and the participations of SMEs and Associations in representation of either feed producers or end-users. TBP is specially qualified for combinatorial analysis, qualitative and quantitative, on complex biological mixtures since it provides a genomic profile of all the species that may be present and a way to produce species-specific molecular probes that can be conveniently used to quantify each of the target species, by real-time, qPCR assays.

Inherent problems concerning the purity of the raw materials used for making compound feed and the lack of available standards of reference had also to be addressed. Raw materials are among those enlisted in the EU Feed materials register.

Fig. 1. Example of raw materials (left) and compound feed samples, flakes or pellets (right)

2. METHODS AND PROCEDURES

Feed-code combinatorial analysis, qualitative and quantitative, is based on the workflow depicted in Fig.2. Four main analytical steps, modules, can be recognized. Module 1 refers to feed sampling, crushing and DNA extraction. For the qualitative analysis (module 2), a single PCR reaction is carried out in order to specifically amplify the targeted DNA regions containing the beta-tubulin genes. Amplicons are then fractionated by capillary electrophoresis (CE) which generates a profile of peaks corresponding to DNA fragments of different length [6]. Each plant species is characterized by a different number of peaks that can vary from 8 to more then 20. The CE output of each species is recorded to constitute a Feed-code data base made up, at present, by reference materials of 31 plant species. The different plant species present in the feed are recognized by comparison of their peaks with those present in the Feed-code database (module 4). For the quantitative analysis (module 3) species-specific Taqman primers and probes for

target plant species were derived from the DNA sequences of TBP-amplification products and, if complying with well defined performace criteria, they are used in real-time PCR assays (qPCR). Quantitative data are then collected and elaborated to release w/w final percentage values (module 4). Technical procedures are described in details in a dedicated manuscript (Braglia et al. submitted manuscript).

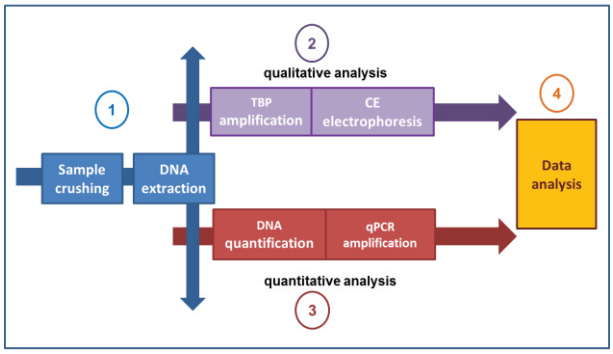

Fig. 2. Workflow of the combinatorial Feed-code analysis

3. RESULTS

3.1. Main biological sources of errors

When dealing with the quantitative analysis of feed samples, one is confronted with several potential sources of uncertainty, distributed all over the experimental procedure (Fig.3). They can be attributed to three major issues: sample representativeness and composition, DNA yield and quality, molecular probe performance and quantitative calibration.

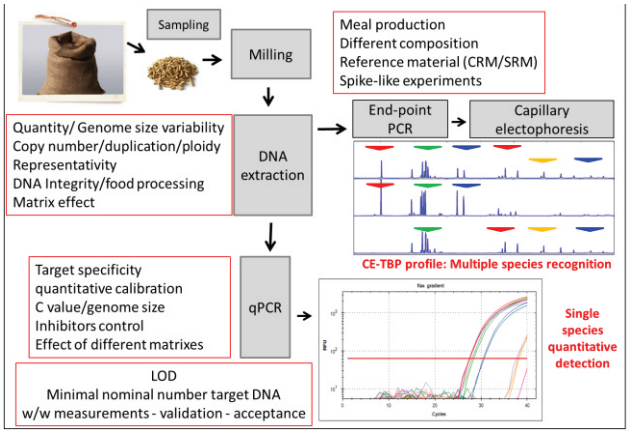

Fig. 3. Diagram showing the main sources of error (red boxes) in the genetic analysis of feed qualitative and quantitative composition

Another important issue is the choice of reference standards needed for the estimation of the relative w/w percentage in the sample. As already mentioned, feed samples are rather complex mixtures made up by different combinations of raw materials that can themselves be present under different forms. This causes a major concern about the availability of representative reference materials that ideally should be as many as the different feed formulations produced. In addition, representative standards of reference should be made starting from pure, uncontaminated raw materials. Both issues are of practical relevance when standard curves are to be produced to quantify the amount of a single ingredient. Dealing with DNA extraction, the problems to face are multiple. First, the DNA extracted should be representative of all the different species present in the feed sample and of their reciprocal ratios within it. This calls for a very efficient, reproducible and repeatable method of extraction. Then, in the perspective of a final quantitative measurement, genome sizes, cell ploidy and DNA content of the different plant materials should be considered as well as the copy number of the target genes. DNA quality, yield, integrity and presence of PCR inhibitors are additional issues to be addressed. Molecular probes should be species-specific but not variety-specific. For the evaluation of probe efficiency and linearity, reference samples at known copy numbers of the target sequences should be prepared

3.2. A multi-step process for probe validation supporting w/w quantitative determination

Within the context of the European project Feed-code, we endeavoured to provide reasonable and practical answers to each of the arguments raised in the previous section (Fig. 4). Given the complexity of the general issue, that is quantification of the feed botanical composition, they do not pretend to be conclusive but they represent more than a good start.

The issue of the reference mixtures was addressed by producing 7 standards of reference (RMs) containing different ratios of a total of 12 different plant species, added as raw materials individually verified for their absolute degree of purity. Thus we chose to discard the unacceptable

idea of having a spiked series of reference standard in a single-component background and that unpractical of having an unlimited number of standards. In a common background made up by the five most common feed components (soft and durum wheat, maize, barley and soybean) we mixed different percentage amount, from 1 to 12%, of either plant raw materials of nutritional relevance (flax, sunflower) or unwanted by some of the Feed-code end-users (cotton, rice, peanut and rapeseed). These reference mixtures, fully mimicking a true compound feed, were useful under different aspects: 1. as *test samples* for sensitivity and repeatability of both qualitative and quantitative analyses; 2. as *control samples*, providing positive and negative controls for all the steps of the analysis; 3. as *standard samples* to provide reference curves for the quantitative analysis. We believe that similar mixtures should be produced as Certified Reference Materials and made available by EC in order to allow more accurate validation to be performed.

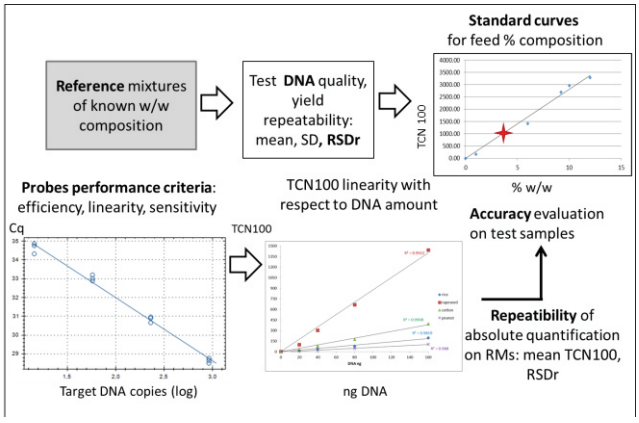

Fig. 4. Schematic representation of the Feed-code procedure leading to the quantitative evaluation of single ingredients of botanical origin present in animal feed samples

An automated method for DNA extraction providing good yield, quality and repeatability values, has been put in place working systematically on individual aliquots of the reference mixtures. DNA amount was determined by fluorometric assay and DNA quality was evaluated photometrically, by UV absorbance reading. A260/280 ratios were always in the range 1.6-1.8, while low A260/280 ratios suggested polysaccharide contamination, practically unavoidable in such samples. Suitability of purified DNA for PCR amplification was evaluated by inhibition test (amplification of a target DNA mixed with the sample DNA). Applicability of the method was tested on eighteen feed samples, confirming the same quality and a yield, ranging from 50 to 180 ng/mg, always granting the feasibility of a PCR approach.

Repeatability was also good when assayed on different feed samples, showing, on average, a Relative Standard Deviation of repeatability (RSDr) of 14,1%. Since DNA yield is variable from feed to feed, the acceptance criterion for the DNA purification procedure was established after repeated testing on one RM, always included as a positive control. DNA yield of this control sample must be between 34 and 56 ng/μl, an amount that accounts for two standard deviations around the mean value. A similar approach could be followed when applying different DNA extraction protocols.

Performance criteria for acceptance of molecular probes specifically targeting single plant species were defined according to both the guidelines of the Codex Alimentarius Commission for the identification and quantification of specific DNA sequences in food [7] and the minimum performance requirements for analytical methods of GMO testing [8]. In accordance, starting from TBP-amplified intron sequences, we have designed, developed and pre-validated plant species-specific Taqman assays for six target plant species: rice, rapeseed, sunflower, flax, peanut and cotton (Braglia et al., submitted manuscript). qPCR assays met the following criteria. Specificity: no-cross reactivity with any of the DNA of the other 30 plant species present in the Feed-code database. Linearity, assessed in the range of 15-1000 target copies, with R2 ≥0.98. Amplification efficiency between 90 and 110%. In order to quantify the absolute number of copies of the target sequence (TCN) present in a given sample, we chose to prepare a set of species-specific plasmid calibrators, which were precisely quantified by digital PCR. Calibrator dilutions at fixed target copy numbers were tested to obtain a 4-points calibration curve. The correlation between measured threshold Cycles (Ct) values and the logaritm of the DNA concentration was determined by linear regression analysis. The repeatability of this absolute quantification method was assayed on different RMs by calculating the mean value of the TCN 100 (TCN normalized to 100 ng of DNA) and the RSDr

values that all scored less than 25%, in compliance with the acceptance criteria suggested for the GMO testing. Such an absolute quantificaton, once opportunely validated, can be applied as such in order to check for presence/absence of plant species whose presence is banned, i.e. toxic *Datura stramonium* seeds.

A further step is required in order to estimate the percent amount of each ingredient in a sample. We produced standards curves by relating the TCN 100 values of each species with the corresponding w/w percentage in the seven reference mixtures. This covered a range of quantification of 1-12% w/w, resulting in standard curves with R2 >0,98 that were used to identify, by interpolation, the amount of the corresponding target species in the feed sample. An example, applied to the analysis described below, is shown in Figure 5. For higher precision, the optimal range should be evaluated case by case in accordance to the expected fraction of the target ingredient in feed. The following criteria were further established: a relative LOD ≤0.5% w/w, and a standard deviation of repeatability ≤ 25%.

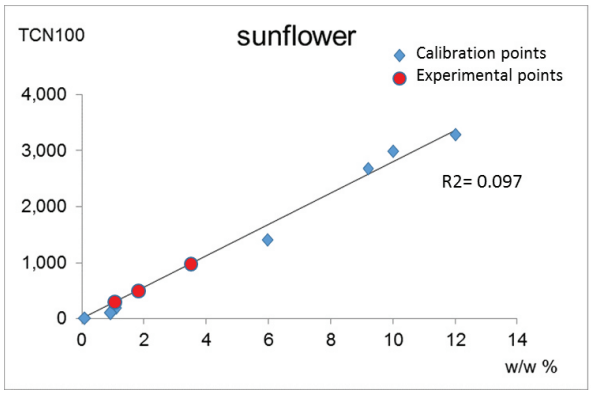

Fig. 5. Calibration curve for sunflower quantification. Experimental points refer to feed samples analysed in Table2

3.3. Control of a feed production line

The Feed-code procedure was thus applied to the analysis of three different lots of the same feed, brought to the lab by a feed producer because two of the samples resulted contaminated by carob, as reported by the optical microscope analysis. It is of relevance to note that the optical microscope (OM) analysis declared the absence of sunflower in any of the feed samples. In addition, a sample of wheat middlings raw material, suspected to be contaminated by rice, was also brought in. The Feed-code qualitative analysis of the same samples, done using the CE for resolution of the TBP-amplified fragments, immediately revealed a relevant contamination with sunflower of the three feed samples, one of which also showed the presence of flax. Contamination of the wheat middlings with barley was also ascertained (Table 1). On the contrary, no peak specific for carob was ever detected.

Table 1. Feed-code qualitative analysis

	sample			
target	Lot1	Lot2	Lot3	wheat middlings
wheat	+	+	+	+
maize	+	+	+	--
barley	+	+	+	+
soy	+	+	+	--
sunflower	+	+	+	--
flax	+	--	--	--
rice	--	--	--	--
carob	--	--	--	--

Qualitative data were confirmed by the related quantitative data obtained with the use of Taqman probes specific for sunflower and flax (Table 2). The assay for barley is not available, at the present.

Table 2. Feed-code quantitative assays

target plant	feed sample	TC/100ng DNA	amount (%w/w)
rice	Lot 1	23.15	0.05
rice	Lot 2	91.06	0.18
rice	Lot 3	50.77	0.10
rice	wheat middlings	16.44	0.03
sunflower	Lot 1	468.05	**1.97**
sunflower	Lot 2	301.56	**1.27**
sunflower	Lot 3	943.77	**3.98**
sunflower	wheat middlings	1.23	0.00
flax	Lot 1	1045.30	**1.46**
flax	Lot 2	6.17	0
flax	Lot 3	6.34	0
flax	wheat middlings	0.00	0

It is also of interest to note that while the optical microscope analysis failed to detect barley in the wheat middlings, in addition to sunflower and flax

in the feed samples, the Feed-code probe specific for rice detected a negligible amount (less than 0,2%) of this species. It is our experience, corroborated by other additional laboratory comparisons, that barley is often mistaken with rice in the OM observations.

3. CONCLUSIONS

The outline and the results of a new method capable of identifying and quantifying the percentage amount of the different plant species present in compound feed samples have been presented. In addition, the Feed-code method allows a rapid, sensitive and reliable assay for controlling the level of purity of the raw materials used to produce compound feed. Diagnostic reliability of the Feed-code method is higher than that achievable with the optical microscope analysis as shown here and as resulting from a recently run European interlaboratory comparison (unpublished). Feed-code is an analytical platform that can be easily upgraded by increasing the number of target plant species of interest. In fact, new genomic profiles of reference can be added to the Feed-code database (at present made up by 31 different plant species enlisted in the European catalogue of feed raw materials) as well as new species-specific molecular probes can be developed and used in quantitative assays.

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REFERENCES

[1] European Parliament and Council of the European Union. Regulation EC No 767/2009. *Off J Eur. Union*, L29, pp. 1-64, 2013.

[2] European Commission. Regulation EU No 51/2013 *Off J Eur. Union* L20/33, 2013.

[3] S. Tavoletti, L. Iommarini and M. Pasquini, A DNA method for qualitative identification of plant raw materials in feedstuff, *Eur Food Res Technol* vol. 229, pp. 475-484, 2009

[4] Voorhuijzen MM, Dijk JP, van PrinsTW, Hoef AMA, van Seyfarth R, Kok EJ, Development of a multiplex DNA-based traceability tool for crop plant materials, *Anal Bioanal Chem* vol. 402, pp. 693-701, 2011.

[5] M. Bardini, D. Lee, P. Donini, A. Mariani, S. Gianì, M.Toschi, C. Lowe and D Breviario ~~(2004)~~ Tubulin-based polymorphism (TBP): A new tool, based on functionally relevant sequences, to assess genetic diversity in plant species. *Genome*, vol. 47, pp. 281–291, 2004.

[6] Gavazzi F, Casazza AP, Depedro C, Mastromauro F, Breviario D Technical improvement of the TBP (Tubulin-based polimorphism) method for plant species detection, based on capillary electrophoresis. *Electrophoresis*, vol. 33, pp. 2840-51, 2012

[7] Definition of minimum performance requirements for Analytical methods of GMO testing. European network of GMO laboratories. ENGL, 2008.

[8] Codex Committee on Methods of Analysis and Sampling. Guidelines on performance criteria and validation on Methods for detection, identification and quantification of specific DNA sequences and specific proteins in food, 2010.