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Manuskript, zu finden in www.fal.de

Published in: Landbauforschung Völkenrode Sonderheft 234, pp. 55-61

Braunschweig
Bundesforschungsanstalt für Landwirtschaft (FAL)
2002

Laboratory evaluation of legume quality

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Abstract

In the EU supported LEGSIL project, the efficient assessment of a wide range of nutritive characteristics in grass and legume forages was achieved by the utilisation of near infrared spectroscopy (NIRS) integrated with conventional chemical laboratory methods and feeding trials. In the agronomic experiments of the project more than 20.000 data concerning nutrient and metabolizable energy content as well as ensilability were ascribed to 3094 samples according to a standard analytical protocol irrespective of whether the forages were grown, harvested and prepared for later analysis in Finland, Sweden, the United Kingdom or Germany.

Furthermore, in an attempt to elucidate the possibilities for intake prediction in legume silage, two alternative methodological concepts previously developed for grass silage were investigated. These concern the silage intake ranking developed in Finland and the approach of intake prediction based on scanning wet silages by near infrared spectroscopy as proposed in Northern Ireland. It was found that neutral detergent fibre as an additional variable improved intake prediction in legume silages by means of the Finnish silage intake index (SII). An even better predictability of intake was achievable through wet silage scanning by NIRS. It is noteworthy that despite the severe limitations for NIRS calibration imposed by the small sample set in our study, 85% of the total variation in intake of legume and grass silage by sheep could be explained by NIRS. This corresponds to the success of the NIRS wet silage intake calibration set up in Northern Ireland on a much larger sample set of only grass silages.

Finally, the view is expressed that advances in dedicated NIRS hardware and software will extend the range of applications into fully automated quality monitoring processes during forage harvesting in the field and silage utilization in dairy operations.

Introduction

It is generally recognized that forage quality is a function of the digestibility and intake of the diet with animal products such as milk, meat or wool providing the most conclusive proof of the value of a given forage. However, the opportunity to test forage quality by means of feeding trials can be considered a rare exception under most practical circumstances. Therefore rational "non animal" forage testing schemes are needed which meet the requirements both of forage breeding stations as well as of dairy farms. While in the first case numerous strains and cultivars need to be assessed for nutritive value as cheaply and efficiently as possible, agricultural enterprises require a reliable integrated scheme from sampling via analysis right up to the use of the analytical data in least cost ration formulation programmes to meet the desired levels of production.

The conventional way of forage testing relies on relating the results of wet chemical analysis via feed tables or empirical equations to the response of the animal . For the practical farming situation even this approach has often proved to be too expensive and time consuming and has been replaced by more or less primitive sensory expert systems based on the visual appearance, physical constitution (grip) or smell which again were related to animal output via feed tables or equations. Such an approach had the benefit of being close "at hand" for the farmer. In the last two decades an unconventional methodological alternative has steadily gained ground which substitutes the human sensory perception of forage quality by opto-electronic sensor measurements in the near infrared part of the electromagnetic spectrum. The first experimental proof of this revolutionary new method was provided by Norris *et al.* (1976) whose publication - viewed from today - represents a true milestone in advancing feed analytical methods into the 21st century. With this method spectral data are usually obtained through reflectance measurements on unfractionated forages. These lend themselves to multivariate statistical prediction of diverse forage quality characteristics and animal performance parameters.

Forage analysis in the LEGSIL agronomy trial: Making rational use of NIRS

Near Infrared Reflectance Spectroscopy (NIRS) is based on molecular absorptions of the OH-, NH- and CH- bonds omnipresent in the organic constituents of forages and other feedstuffs. Due to the enormous complexity of these highly overlapping, partially

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repetitive absorptions and due to their interactions with the particular physical status of each sample at the time of measurement, the implicit use of Beer's Law in applying NIRS for quantitative purposes depends on statistical modelling (cf. Shenk and Westerhaus, 1994). This requires special sets of samples typical of a given product to serve for calibrating NIRS instruments. An example of such a calibration set may be provided by a set of forages dried and ground according to a specified protocol with known contents of chemical constituents, chemical fractions or quality characteristics such as water soluble carbohydrates, crude fibre or *in vitro* digestibility of the organic matter. NIRS equations developed as prediction models for any given product and constituent on a particular instrument perform adequately as long as applied to samples which are essentially comparable to those utilized in the calibration (modelling) process. Based on such a rationale, forage quality analysis in large sets of samples can be performed efficiently by calibration in a small subset of a large total set of samples. Once the characteristics of the subset are known and the NIRS prediction equation is established, the latter can legitimately be applied to the total sample set. By allowing the complete analytical characterisation of large trials without the need for a complete coverage of all samples by conventional analytical means, this procedure permits enormous savings in laboratory costs without loss of analytical information.

A case in point is provided by the agronomy trial of the LEGSIL project which aimed at evaluating the agronomic and nutritive characteristics of five different legume species grown in monoculture and in mixture with grass ranging from the south-west of the British Isles to the East into Northern Germany and up to the North East via South Sweden to locations near the Northern, Central and Southern Baltic coast of Finland (Halling *et al.* 2002). This trial was conducted over 12 locations and included from two to three cuts in two main harvest years with three field replicates at each location. The information to be gained from the trial included not only the content of nutrients and metabolisable energy, but also the ensilability of the forage samples. Thus a considerable amount of analytical effort was expected to be required for a complete characterisation of the samples. The usual practice of analysing the samples in national laboratories according to sample origin would still have resulted in a given laboratory having to process between 432 and 973 samples (Table 1) for a considerable number of parameters.

Table 1:
Number of samples analysed for diverse quality characteristics in LEGSIL agronomy trial

	Country origin of samples				
Harvest Year	UK	D	S	Fin	Total
1998	488	324	431	108	1351
1999	485	507	427	324	1743
Total	973	832	858	432	3094

Moreover, such an approach would have posed the risk of systematic deviations between laboratory methods inflating site effects, despite confirmation of previous satisfactory harmonisation of wet chemical procedures for silage analysis among laboratories participating in the LEGSIL project (Paul et al. 1999). In contrast , within a well coordinated investigation quality assessments by NIRS do not pose a problem either in terms of analytical turnover or with regard to interlaboratory errors. Due recognition of these advantages led to the decision to make use of NIRS for multiple forage analysis within the 3094 samples of the LEGSIL agronomy trial by establishing NIRS prediction equations for each required quality characteristic in a representative subset of samples and by applying these to the total sample set.

The procedure by which the subset of samples for NIRS calibration were chosen made use of an algorithm introduced by Shenk and Westerhaus (1994) for selecting spectra of representative samples from a data file containing the spectra of the total set of samples. This centers on the ranking of all spectra according to the Mahalanobis distance from the average spectrum of the file. In this way 394 samples (i.e. 12.9% of the total set) were selected. This selection was achieved by choosing in stepwise fashion a NIRS calibration set across samples of the 1998 harvest from the four participating countries and by updating this set with suitable samples from the 1999 harvest. Judged by the Mahalanobis distance between spectral data it was shown that the resulting broad based European calibration set included more suitable information for making a general NIRS model adapted to all the various sample sets from the four participating countries than a relatively narrow one based on samples from one country alone. The respective "wet chemical" laboratory methods were performed at the former FAL Institute of Forage and Grassland Research on the selected calibration samples and used as straightforward calibration inputs. For uniform quality assessment of the samples

from both harvest years across countries, the European NIRS equations were then employed.

For a complete evaluation of the trial, analytical information was required a) on the content of metabolisable energy, b) on the content of crude nutrients according to the Weende method and c) on ensilability.

Considering the crucial relevance of metabolisable energy (ME) in forage quality evaluation and the relative paucity of such data for forage legumes in Northern Europe a major effort was undertaken to set up a satisfactory and promising strategy using a novel approach. Hence, its details are outlined in the following. The regression equation for ME content of grass and grass products proposed by Weissbach et al. (1999) was utilized in this strategy. It possesses the inherent advantage of being universally applicable to grass, grass silage and hay, even if produced from extremely late cuts, but it has not yet been extended to forage legumes. Its general format is based on the content of enzymatically insoluble organic matter (EIOM; in German EULOS), crude protein and crude ash. For these parameters the above mentioned European NIRS equations had been developed.

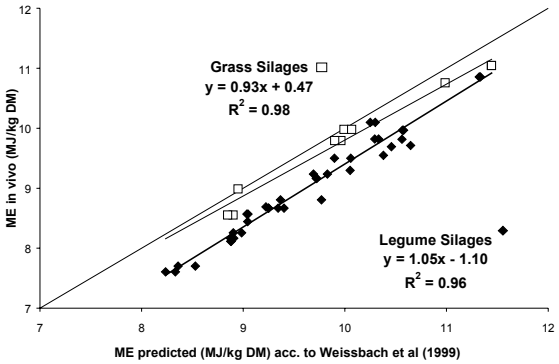

Figure 1:
Performance of prediction equation for content of metabolisable energy (ME) developed by Weissbach et al. (1999) with silages prepared from grass and legumes

The validity of this regression equation for grass silage was confirmed in the LEGSIL feed evaluation experiments of FAL on grass and legume silages performed on sheep (Paul *et al.* 2002) (Figure 1). As was expected, the prediction of ME content of silages from forage legumes by means of this equation resulted in a systematic error, the extent of which provided an opportunity to calculate ME correction equations not only for pure legumes but through interpolation also for mixtures with varying composition of grasses and legumes. As a consequence a series of four regressions intended for use with a) pure and dominantly grass (> 75% grass), b) mixtures with 50% - 75% grass, c) mixtures with 25% - 50% grass and e) pure and dominantly legume (< 25% grass) were computed (Figure 2).

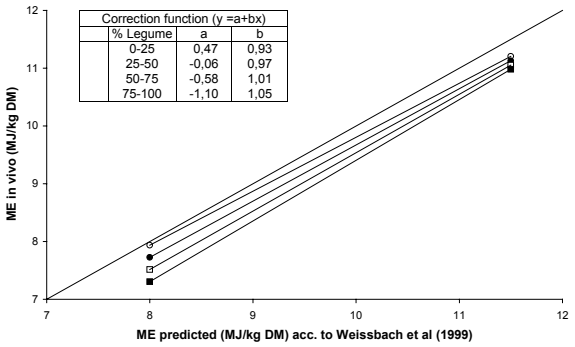

Figure 2:
Content of metabolisable energy according to Weissbach et al. (1999): Derivation of correction functions according to legume content of samples

As the calibration and cross-validation statistics appeared satisfactory (Table 2), the NIRS equations were accepted for use on all 3094 samples of the LEGSIL agronomy project. ME contents were computed by means of the NIRS predictions of the three variables and corrected according to the legume proportion of each particular sample. This finally resulted in ME contents of grasses, legumes and their mixtures in line with the results of the LEGSIL feed evaluation experiment with sheep (Paul et al. 2002)

Table 2:
NIRS calibration and cross validation statistics for variables determining content of metabolisable energy (according to Weissbach et al. (1999)

	EIOM g /kg OM	Crude Protein % of DM	Crude Ash %of DM
N	384	363	381
RSQ	0.96	0.99	0.88
SEC	15.04	0.58	0.53
SECV	16.81	0.65	0.64

In order to make use of the Weissbach model for the evaluation of the 3094 samples of the LEGSIL agronomy experiment, NIRS equations were developed for the prediction of the content of enzymatically insoluble organic matter (EIOM), crude protein and crude ash as determinants of ME content based on the samples in the calibration set.

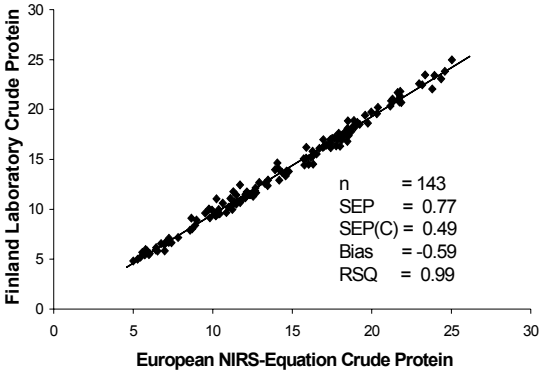

Figure 3:
Analytical performance of crude protein assessment by European NIRS equation in forage samples from Finland

The assessment of crude protein may serve as an example of how NIRS equations developed across the calibration samples from the four countries performed in comparison to conventional analytical chemistry. There was generally a high degree of correspondence between the crude protein data predicted by the European NIRS equation for given samples and the equivalent values obtained by the conventional procedure. This was typified through a comparison of the crude protein predictions by NIRS (based on the European NIRS equation established on crude protein determination at the FAL laboratory) and crude protein determined conventionally at the laboratory of Helsinki University on the samples from Finland (Figure 3). The small systematic error (bias) and the small random error (SEP(C) observed under these conditions are equivalent to the usual level of interlaboratory (bias) and intralaboratory (random) errors in the reference method. This provides proof of the excellent correspondence achieved between the European NIRS calibration and the wet chemical assessment of crude protein according to Kjeldahl. In contrast to this, the methods for the assessment of OMD in vitro as practised in the different LEGSIL partner laboratories followed different protocols and did not show the same degree of interlaboratory correspondence as the Kjeldahl method (results not shown). In this case, the utilization of the European NIRS calibration as based on one single reference lab contributed considerably to the harmonisation of the OMD data across the four different LEGSIL partner countries.

Silage intake prediction in the LEGSIL sheep feeding trial: Tapping the potential of NIRS

The assessment of feed quality remains incomplete when attention is not paid to the voluntary intake of that feed by ruminant animals. However, as pointed out by Mertens (1994), "The number and complexity of factors affecting intake make the measurement of intake potential of a forage difficult to accomplish and interpret." He also points out that the animal and feed factors regulating intake are to some extent inseparable and that therefore "the interaction of the forage, animal, and feeding situation precludes the prediction of actual intake based solely on feed characterisation". These considerations must serve as a warning to experimenters who risk severe errors when attempting to extrapolate or draw sweeping conclusions from limited feed evaluation experiments. Yet the search for feed factors regulating intake within a well defined experiment on different species of forage legumes and grass like the LEGSIL feed evaluation study by Paul et al. (2002) seems legitimate. In this case the range from low to high quality forages fed to mature sheep wethers with constant, moderate energy demand resulted in large differences in voluntary intake between roughages. It may be assumed that in this experiment – typical for any comparison between grass and legume species with sufficient variation in energy content - effects of energy availability, physical structure and palatability on voluntary intake played an important role so that on the one hand a reliable relative ranking of these species in terms of intake potential, similar to the French fill values (INRA 1989) was possible. On the other hand, the factors responsible for intake variation in these forages under the conditions of the experiment could be investigated and laboratory methods for their assessment tested . The latter appeared legitimate, particularly because the potential of two alternative methods was tested against the background of the same set of samples. In our case recently proposed approaches for the prediction of intake in grass silages as developed in Finland and in Northern Ireland were compared for their suitability for intake prediction.

The Finnish system of ranking grass silages for intake published by Huhtanen *et al.* (2002) is based on three factors, i.e. D-value (D), content of total fermentation acids (FA) and the proportion of ammonia nitrogen of total forage nitrogen (NH_3-N). The Silage Intake Index (SII) is computed in the following way:

$$SII = 100 + 0.151\,(D - 690) - 0.000531\,(FA^2 - 6400) - 4.7650\,[Ln(NH_3\text{-}N) - Ln(50)]$$

The correlation coefficients characterising the relationships between these variables and dry matter intake for the samples studied by Paul *et al*. (2002) are given in Table 3. The independent variables D-

value and NH_3-N were more closely related to intake in the silages prepared from grass than in those from grass and legumes. The opposite was true for the total fermentation acids. As was to be expected, the Finnish Silage Intake Index had a closer relationship to intake when grass silages alone rather than grass and legume silages were included in the sample set (Table 3). Among the variables known to influence intake, neutral detergent fibre (NDF) is often regarded as one of the routinely measured essential feed factors for estimating the filling effect of a forage. So it appeared appropriate to also consider NDF in this investigation. The addition of NDF did indeed improve markedly the relationship of SII with intake in the mixed sample set of grass and legume silages. In contrast the addition of NDF did nothing to improve the relationship of SII with intake in the grass silage sample set.

Table 3:
Variables for rank prediction of grass silages in voluntary intake (according to Finnish Silage Intake Index (S I I)): correlation coefficients with intake observed in LEGSIL sheep feeding trial

Variable	Silage from:	
	Grass only	Legumes + Grass
D value (g DOM / kg DM)	+ 0.69	+ 0.47
FA (g / kg DM)	+ 0.39	+ 0.55
NH_3-N (g / kg N)	- 0.70	- 0.15
S I I	+ 0.87	+ 0.36
S I I + NDF	+ 0.88	+ 0.88

The above results should not be misinterpreted as the outcome of an unfair test of the Finnish silage intake ranking scheme for grass silages. But faced with a situation where an increased utilization of forage legume silage in dairy rations might take place at the farm level, the limited applicability of the SII to pure legume silages as well as silages prepared from mixtures of grass and legumes is important. Additionally, it is worth noting that NDF can improve the performance of the Finnish SII in samples containing significant proportions of legumes without impairing its performance with pure grass silages.

Taking account of all the variables, and possibly also NDF, to be assessed in the laboratory for the prediction of voluntary intake according to SII , it is understandable that less laborious and potentially more accurate methods are still sought. Since the first report of a correlation study on NIR spectra of forages and voluntary intake by Norris *et al.* (1976) the potential of NIRS has been confirmed in several reports. . But most studies have been directed towards the goal of predicting intake based on NIRS measurements in dried silage samples. In NIRS studies - as with conventional analytical techniques - some method of dehydration is usually practised, because the bulk of energy and nitrogen containing nutrients is either part of or associated with the cell wall fraction rather than with the cellular contents. Furthermore water dominates the NIR spectrum of undried, fresh silage as a consequence of both its high content in fresh forage and its very high absorbance in the near infrared. This is exemplified by the spectra of undried and dried lotus silage (see Figure 4). But NIRS protocols for the analysis of dried silage suffer from the disadvantage of a lengthy drying process during which volatile constituents of the silages, such as fermentation acids, alcohols and ammonia (compounds which may have functional relationships with intake), are liberated and lost. Therefore the approach used by Gordon *et al.* (1998) in the very systematic investigation on the largest set of silage samples yet studied in a NIRS calibration experiment on wet silages appeared attractive. It was used as a basis of comparison with the much smaller study reported here.

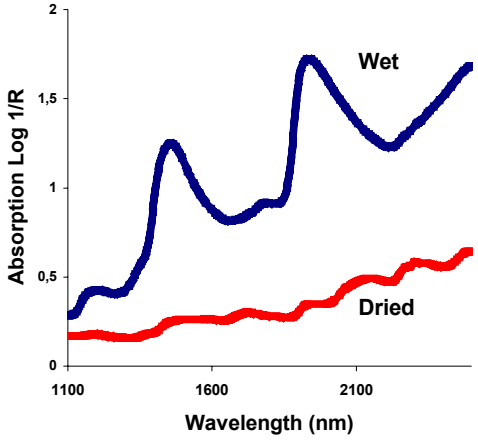

Figure 4:
NIR diffuse reflectance spectra of dried and wet silage prepared from *Lotus corniculatus* forage

The significance of whether silage in NIRS analysis is scanned as wet or dry may be appraised from the results of a principal component analysis (PCA) of the spectra obtained on the set of legume and grass silages of the LEGSIL sheep feeding trial. PCA was used to define absorbers (factors) which were underlying the NIR spectral variation of this set of samples when scanned either dried or wet. For each forage quality parameter, the three best suited

absorbers were identified by correlation analysis. As far as wet silage was concerned, the multiple correlation between OMD *in vivo* and the three best NIR absorbers for OMD explained only 52% (RSQ x 100) of its variation in this sample set (Table 4). However, in the same sample set scanned in the dried state, the multiple correlation between the same data for OMD *in vivo* and the three best NIR absorbers for OMD rose considerably so that 74% of its variation was explained. An even more dramatic improvement in the same direction took place for crude protein content. In contrast to this, dry matter intake (DMI) by sheep (and NDF content) was almost equally well explained in wet and dried silage by three PCA factors. These results indicate that NIRS scanning of wet silages lends itself more to the prediction of intake than to the prediction of OMD. But it is well known that complex feed characteristics require complex NIRS regression models, so that OMD and intake are expected to be explained more satisfactorily by more than just three absorbers. Taking the results of Gordon *et al.* (1998) as an example, up to 16 PCA factors were selected for use in predicting OMD and intake.

Table 4:
Principal component analysis (PCA) of LEGSIL silage NIR spectra: proportion of explained variation of diverse forage quality parameters by optimal 3 PCA factors

Quality Parameter	Silage scanned: Wet		Dried	
	RSQ	Rank	RSQ	Rank
DMc	.93	1	-	
DMI	.70	3	.74	3
OMD in vivo	.52	4	.74	3
NDF	.87	2	.88	2
Crude Protein	.53	4	.95	1

Also, in the same experiment it was shown that a) transformation of spectral data, such as derivatisation, allied to scatter correction procedures generally improved prediction accuracy and b) modified partial least square (MPLS) factors were superior to PCA factors as variables in NIRS regression analysis for predicting both intake and OMD. When calibrations were developed both for wet and dried silages according to the above mentioned findings, the lesser number of samples of the LEGSIL sheep feeding trial enforced regression models with reduced complexity than developed by Gordon *et al.* (*l.c.*), i.e. with only 6 rather than up to 11 MPLS factors. Nevertheless, in cross validation of the 6-factor models for DMI and OMD in wet silage, standard errors (SECV) of 0.12 DMI (kg / animal / day) and 2.32 OMD (%) were observed with 85% and 88% of the variation in DMI and OMD explained ((1-VR) x 100) (Table 5). The corresponding evaluation for dried silage resulted in a significantly better fit of predicted vs. observed data for OMD and a similar tendency for DMI.

Table 5:
Performance statistics of NIRS regressions* for prediction of dry matter intake and organic matter digestibility in silages scanned wet and dried

	Wet silage		Dried silage	
	SECV	1-VR	SECV	1-VR
DMI**	0.12	.85	0.11	.88
OMD***	2.32	.88	1.63	.94

* Data transformation: 1,4,4,1; MPLS regression analysis without outlier removal
** Dry matter intake: kg / animal / day
*** Organic matter digestibility: %

Considering the smaller data base, the fewer factors in the regression equation and the increase in population heterogeneity in our sample set of grass and legume silages compared to the NIRS calibration experiment solely on grass silages by Gordon *et al.* (*l.c.*), inferior results in terms of the degree of fit achieved were expected for our calibrations. However, similar performance statistics were found for the wet silage calibrations in both experiments.

Outlook

The present paper has demonstrated the potential of near infrared spectroscopy as an efficient opto-electronic tool which allows the assessment of multiple forage quality parameters through one single measurement with great ease of sample preparation, non-consumption of sample and high speed of operation. Its utilization is of paramount importance for rational feed and forage testing laboratories. Recent advances in dedicated NIRS hardware and software will extend the range of applications even further. These are due to spectrometers which allow parallel rather than sequential scanning and to regression analytical techniques for dealing with non-linearity in the opto-electronic expression of compositional variation of feedstuffs. Consequently, we will be able to fully realise the inherent capacity of the NIRS technique with its integration in a fully

automated process from harvesting, sampling, minimal preparation to the analysis of forages in real time on forage harvesters (Paul *et al*. 2000). Similarly the introduction of NIRS elements into precision agriculture in dairy operations will prepare the ground for a better monitoring of feed ration optimisation than was possible before.

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Summary of plenary discussion

1 Effects of fermentation quality

The possible effects on intake of silage fermentation quality, as reflected by high ammonia-N concentrations in the lucerne silages and concentrations of acetic acid were queried. It was pointed out that the ammonia figures would include some N from the additive, which contained both hexamine and sodium nitrate. Although this would have increased ammonia for all silages, it would not give a differential effect between the crop species.

The author considered that differences in silage fermentation characteristics were not having a major impact on intake with this population of silages. Some 85% of the variation in intake could be accounted for by digestibility.

2 Intake of lotus

In relation to the reason for the high intake of lotus silages, the author referred to rumen degradability studies carried out on these silages. These had shown that DM degradability was more rapid with lotus than the other legume silages and this was probably responsible for the high intakes.

Possible effects of tannins on N digestion were queried. Tannins had not been determined in these experiments. G. Broderick indicated that tannin concentrations were generally lower in Lotus corniculatus than in other tannin-containing legumes and did not think that major effects were likely. N. Nilsdotter-Linde referred to work in Sweden. She had shown large differences between varieties in tannins, but that values for Leo, the variety used in LEGSIL, was generally low. There was, however a negative relationship between tannin content and rate of protein degradation in the rumen, despite tannin concentrations being generally low.

Reporter: R.J. Wilkins