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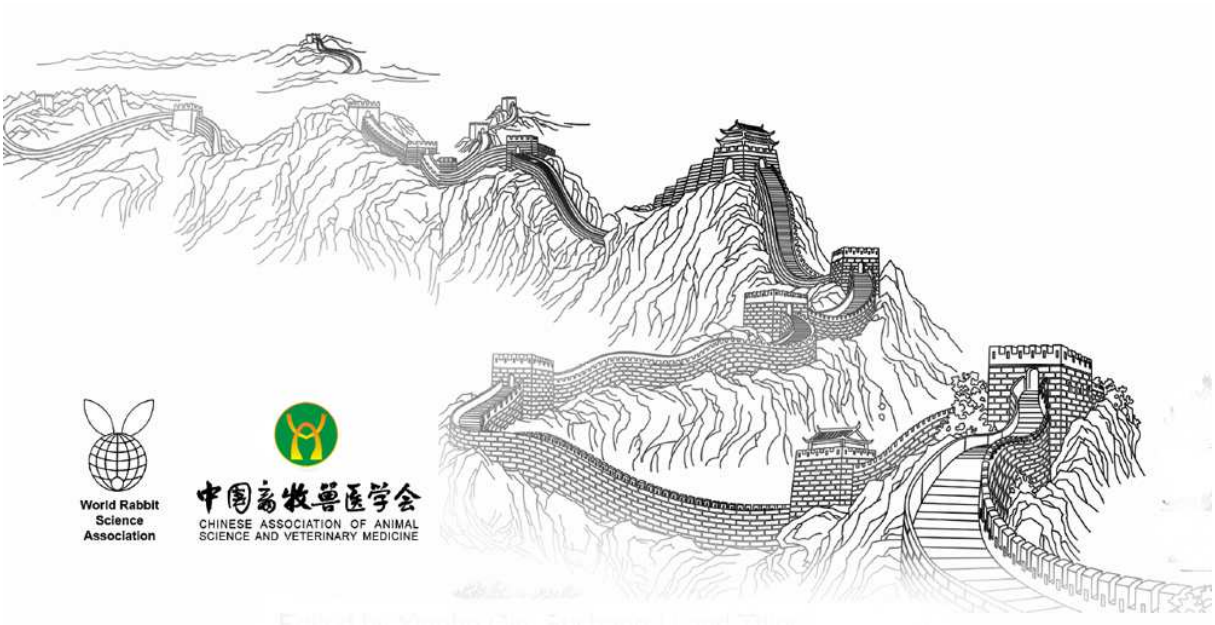

COMPARISON OF TWO METHODS RELATED TO THE LIGNEOUS FRACTION ANALYSIS

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ABSTRACT

Fibers — especially the ligneous fraction of fibers — are a key factor for ensuring rabbits' digestive health. Several methods are designed to analyze the lignin content. The most commonly used ones are the Van Soest's method and the "direct" method related to insoluble lignin in H_2SO_4. Therefore this study aims to compare these two approaches in order to determine which one is the most relevant for an accurate determination of fiber supplies. The results sometimes show substantial deviations between the two methods, especially when it comes to grape by-products (up to 7.7 additional points are reached when applying Van Soest's method). Furthermore, when using a mix of fibrous raw materials such as those contained in Lapilest®, the insoluble lignin H_2SO_4 method achieves the best additivity among the unit values related to each raw material. Additional analyses obtained with Van Soest's method indicate an overestimation of the ADL value as the process leads to the formation of complexes between lignin and phenolic compounds of the tannin type.

Key words: analysis, lignin, Van Soest, insoluble lignin in H_2SO_4, polyphenols, tannin

INTRODUCTION

Fibers are one of the major component of a rabbit feed. In addition to assuming the role of nutrient, they have an effect on intake level and the transit speed of feeds. They also act as a major substrate for the caecal microbiota (Combes et al., 2013). Their influence on the management of sanitary risks is widely recognized, keeping in mind that their effects differ depending on the fractions considered (lignin, cellulose, hemicellulose, pectin) (Gidenne, 2014). Several studies have already shown the positive impact of lignin concentration in feed on digestive mortality (Perez et al., 1994; Colin et al., 2007).

It is thus essential that the fibers content of raw materials be well characterized, especially lignin, so that the accuracy of the nutritive value of feed can be improved (Gidenne, 2000). To this end, several different methods apply to the quantitative analysis of fibers. The most common methods in feed nutrition are Van Soest's one and the method of the insoluble lignin in H_2SO_4. In the first instance, this study aims to compare these two methods by respectively applying them on five raw materials and on a fibrous mix, the Lapilest®, used in rabbit feeds. It also aims to validate the additivity of the analysis results; this is a key criterion for determining the efficiency of a method. Additional analyses are then performed on phenolic compounds for a better understanding of the differences that are observed.

MATERIAL AND METHODS

Analysis related to the different raw materials and the Lapilest® fibrous mix

Five different raw materials and a commercial fibrous mix made up of these 5 raw materials —i.e. a total of 56 samples— are submitted to various kind of analysis: moisture (at 103°C NF ISO 6540), NDF, ADF and ADL fibrous fractions (Van Soest, NF V 18-122) and the insoluble lignin in H_2SO_4 (V 18-115).

Additivity among the methods of analysis

Van Soest's ADL and the lignin H_2SO_4 methods are applied to each of the five raw materials composing the Lapilest®, and on the fibrous mix stemming from the same batches of raw materials. The weighed sum of these raw materials is compared to the analytic value of the Lapilest®.

Analysis of the phenolic compounds

The phenolic compounds of the raw materials contained in Lapilest® are quantified by using the high performance liquid chromatography, combined with the mass spectrometry (UPLC-DAD-MS). These analyses are carried out at the Polyphenols Biotech Laboratory (Villenave d'Ornon, France).

RESULTS AND DISCUSSION

Comparisons drawn from analyses resulting from the two methods

Chemical analyses show that the raw materials examined have quite different fibers profile (Table 1). The deviations revealed between the two methods of ligneous residue analysis (Van Soest's ADL, H_2SO_4 lignin) vary widely depending on the raw material that is considered (Figures 2 and 3): beet pulp, a little ligneous (ADL < 5%), has a small deviation (an average of 0.17 pt). Sunflower hulls and apple pomaces, much more ligneous (ADL between 15 and 25%) have also small deviations (+ 0.88 pt and - 0.54 pt resp.). However, grape by-products, very ligneous (ADL>30%) and the Lapilest® (ADL=24.95%) show significant deviations (an average of + 7.04 pts). Apart from the apple pomace, Van Soest's ADL method shows an overestimation of the lignin value when compared with the insoluble lignin in H_2SO_4 method.

Table 1: Fibers content of the raw materials and the Lapilest®

	Moisture %		NDF %		ADF %		ADL %		H_2SO_4 Lignin %	
Raw materials (number)	Moy	*e-t*	Moy	*e-t*	Moy	*e-t*	Moy	*e-t*	Moy	*e-t*
Beet pulp (n=9)	10.9	*1.07*	39.8	*1.26*	20.5	*1.81*	1.7	*0.45*	1.5	*0.59*
Sunflower hulls (n=5)	8.8	*1.93*	74.7	*1.39*	56.6	*0.98*	19.6	*0.72*	18.7	*1.57*
Apple pomace (n=8)	8.7	*0.69*	62.8	*1.58*	56.0	*2.43*	22.8	*2.03*	23.3	*3.09*
Grape pulp (n=17)	8.2	*2.57*	56.8	*7.00*	49.0	*4.82*	34.3	*4.18*	26.6	*4.40*
Grape seeds (n=7)	8.9	*1.72*	62.1	*5.83*	52.1	*5.38*	42.3	*4.46*	36.7	*5.33*
Lapilest® (n=10) *	10.7	*1.08*	56.8	*1.15*	44.8	*1.49*	25.0	*3.34*	17.6	*2.12*

* Lapilest®: mix of grape pulp, beet pulp, sunflower hulls, grape seed and apple pomace (in decreasing order of incorporation)

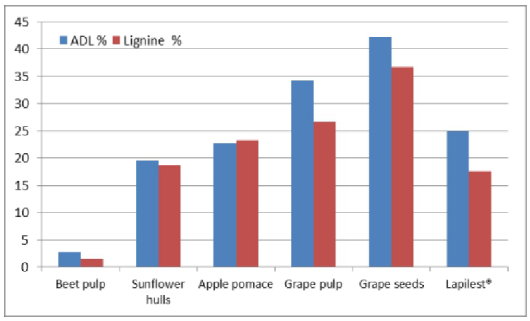

Figure 1: Levels of ADL and Lignin H_2SO_4

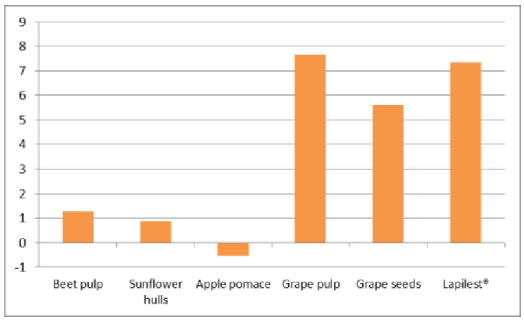

Figure 2: Difference (ADL-Lignin H_2SO_4)

Additivity of the two methods

As seen previously, ADL results turn out to be higher than those obtained with the H_2SO_4 lignin method (Table 2). Figure 3 implies that the weighed sum of the H_2SO_4 lignin values is similar to the H_2SO_4 lignin measured on the Lapilest® (15.9% and 16.0% resp.). This does happen when Van Soest's method is applied (21.7% and 30.4%% resp.). This deviation, which stems from the grape by-products, well reflects an overestimation when Van Soest's method is applied. Conversely in the framework of the H_2SO_4 lignin method, the results that are obtained faithfully reflect the actual unit values of each raw material.

Table 2: Comparative measures of the ADL and H_2SO_4 lignin levels

	ADL Van Soest	Lignin H_2SO_4	ADL - Lignin
LAPILEST®	30.4	16	14.4
Beet pulp	1.3	1	0.3
Sunflower hulls	20.6	19.1	1.5
Apple pomace	20	18.7	1.3
Grape pulp	33	20.6	12.4
Grape seeds	44.3	33.8	10.5

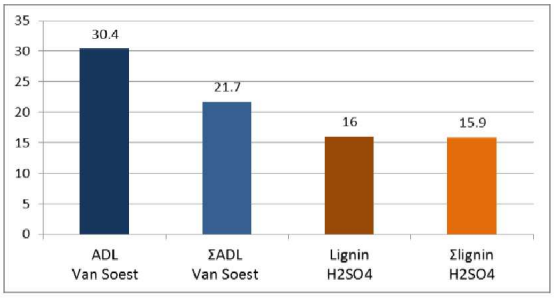

Figure 3: ADL and H_2SO_4 lignin percentage in the Lapilest® (direct measure and total amount of the measures from the components)

As for the Lapilest®, a rabbit feed that contains grape by-products is likely to show a lignin deficit if the characterization of its raw materials is based on Van Soest's ADL method. This may strongly impact the health status of rabbits.

Analysis of the phenolic compounds in the raw materials

From bibliographic data, it appears that the neutral detergent step applied by Van Soest's method (Figure 4) leads to the formation of proteins-tannins complexes in the presence of tannins (phenolic compounds) (Terrill *et al.*, 1994). In addition, the various stages of high temperature drying (3 times for 3 hours at 103°C) (Figure 4) result in increasing the polymerization of these tannins and their complexation with other cellular components (fibers, proteins) which are not totally solubilized during the acid attacks (acid detergent and H_2SO_4) (Terrill *et al.*, 1994). Therefore the ADL residue resulting from the application of Van Soest's method could contain lignin and polymerized tannins (Godin, 2011) thereby leading to an overestimation of the lignin level.

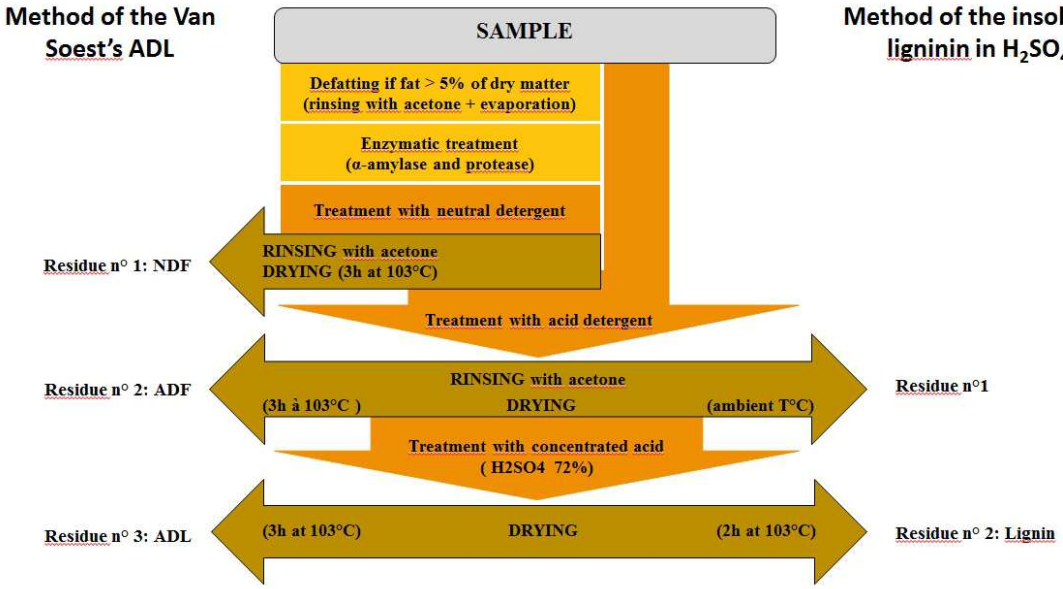

Figure 4: Comparison between Van Soest's method (NF V 18-122, AFNOR, 1997) and the insoluble lignin in H_2SO_4 method (V 18-115, AFNOR, 1993)

Analysis of the phenolic compounds shows that grape by-products are the richest in polyphenols (3380µg/g in the grape pulp and 5208µg/g in the grape seeds). They also contain a high proportion of flavonoids (87.7% and 92.9% resp.) — the flavonoid sub-family contains tannins among other compounds (Table 3). The sunflower hulls show an intermediate level of polyphenols (1042 µg/g) which do not belong to the flavonoids sub-family. (602 µg/g) The apple pomace also contains polyphenols yet to a lesser level, while the beet pulp does not show any of them.

Table 3: Quantification of the phenolic compounds in the raw materials

	Grape pulp	Beet pulp	Sunflower hulls	Grape seeds	Apple pomace
Phanolic acids (µg/g)	420	0	1042	370	105
Flavonoids (µg/g)	2960	0	0	4838	497
Total (µg/g)	3380	0	1042	5208	602

The grape pulp and the grape seeds ─ which contain the highest level of flavonoids ─ show the most significant deviation between ADL and lignin H_2SO_4 values. This result lends support to the hypothesis that an interaction with polyphenols actually occurs during the analysis, especially regarding flavanoids (tannins). Other bibliographic data conjecture an insolubility of the nitrogen matters in the detergents used with Van Soest's method. These would be thus found in ADL residue (Koné, 1987). It could be relevant to lead further analyses for measuring the nitrogen level in the raw materials and the intermediate residues. However, according to other studies, unlike our results, the ligneous fraction obtained through Van Soest's method could stem from the sulfuric acid attack resulting in weakening or solubilizing a part of the lignin (Giger 1987). However, these results are often based on forages, less ligneous and which have a low concentration of polyphenols. Therefore further analyses need to be carried on other raw materials.
This study constitutes a first step for a better understanding of the phenomena; it could explain deviations in the different methods of analysis.

CONCLUSION

This study reveals deviations in the results between two methods of analysis respectively undertaken on different kinds of raw materials and their mix. In the case of grape by-products, Van Soest's method leads to an overestimation of their ligneous fraction when compared with the method of the insoluble lignin in H_2SO_4. This is due to the formation of complexes with polyphenols (tannins). To characterize the ligneous fraction in raw materials that are rich in polyphenols and in rabbit feeds, our conclusion is that the insoluble lignin in H_2SO_4 method is more relevant than Van Soest's one. Yet our work shows that Van Soest's ADL method may still be pertinent for analyzing the content of raw materials that are slightly ligneous and that have a low level of polyphenols.

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Fibers — especially the ligneous fraction of fibers — are a key factor for ensuring rabbits' digestive health. Two main methods are used to analyze the lignin content : Van Soest's method and the "direct" method related to insoluble lignin in H2SO4. This study aims to compare these two approaches in order to determine which one is the most relevant for an accurate determination of fiber supplies.

Material and methods

1 Analysis related to the different raw materials and the Lapilest® fibrous mix
Five different raw materials and a commercial fibrous mix made up of these 5 raw materials —i.e. a total of 56 samples— are submitted to various kind of analysis: moisture (at 103°C NF ISO 6540), NDF, ADF and ADL fibrous fractions (Van Soest, NF V 18-122) and the insoluble lignin in H2SO4 (V 18-115).

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Van Soest's ADL and the lignin H2SO4 methods are applied to each of the five raw materials composing the Lapilest®, and on the fibrous mix stemming from the same batches of raw materials. The weighed sum of these raw materials is compared to the analytic value of the Lapilest®.

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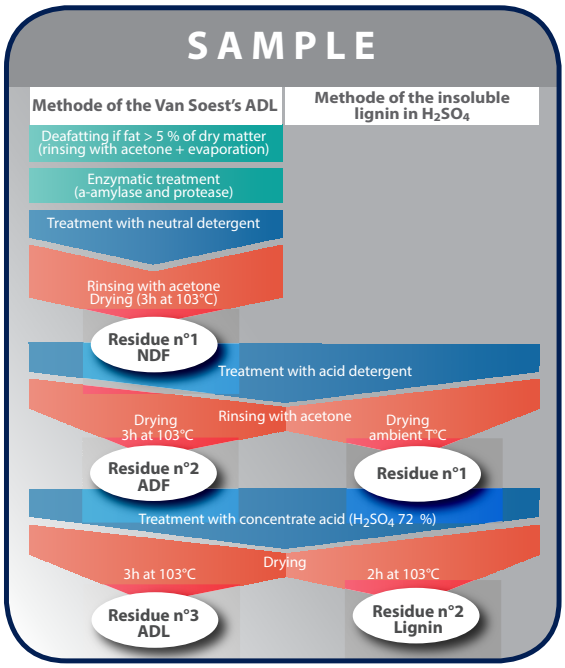
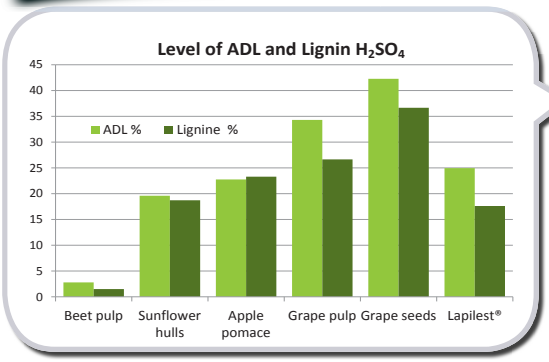
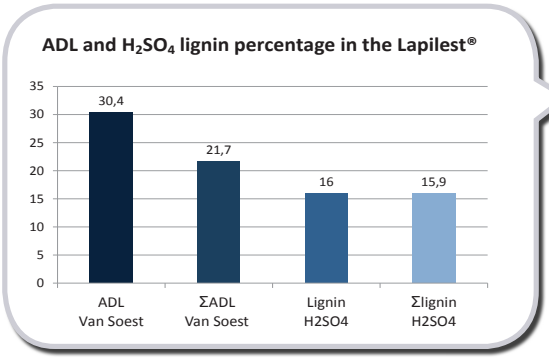


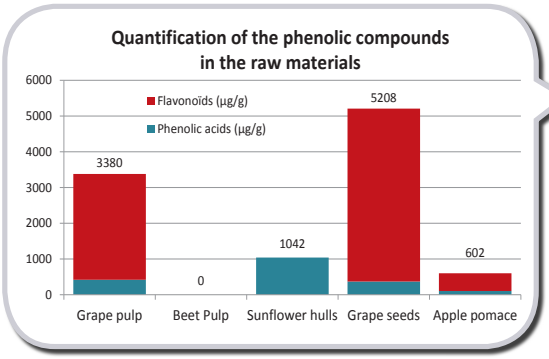
Results and discussion



The deviations revealed vary widely depending on the raw materials:
- Small deviations for some raw materials (beet pulp, Sunflower hulls or apple pomaces (-0.54 to 0.88 pt).
- Higher deviations for Grape by-products and the Lapilest® (average of + 7.04 pts).



Additivity is demonstrated with the H2SO4 lignin values is similar to the H2SO4 lignin really measured on the Lapilest® (15.9% for the weighed sum of the raw materials and 16.0% really measured).
Van Soest's method showed an overestimation of lignin (21.7% with the weighed sum and 30.4%% measured).



Two reasons explain the overestimation of the ligneous fraction from Van Soest's method, especially on raw materials containing a lot of polyphenols (Flavonoïds) like grape by-products:
1/ the formation of proteins-tannins complexes in the presence of tannins (phenolic compounds in flavonoids sub-family) during the neutral detergent step.
2/ the polymerization of these tannins and their complexation with other cellular components (fibers, proteins) because of the various stages of high temperature drying.

This study reveals:
- **Deviations in the results between two methods** of analysis
- In the case of **grape by-products, Van Soest's** method leads to an **overestimation of their ligneous fraction** when compared with the method of the insoluble lignin in H2SO4, due to the **formation of complexes with polyphenols** (tannins).
- To **characterize the ligneous fraction** in raw materials that are rich in polyphenols and in rabbit feeds, our conclusion is that the **insoluble lignin in H2SO4 method is more relevant.**
- Yet our work shows that **Van Soest's ADL method may still be pertinent for analyzing the content of raw materials that are slightly ligneous or that have a low level of polyphenols.**

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