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Original article

Effect of different drying systems for the conservation of olive leaves on their nutritive value for ruminants

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Abstract – Leaves obtained from olive trees (*Olea europaea* L.) were stored under various conditions for periods up to 42 months. Duration of storage had a marked effect on crude protein digestibility of leaves when fed to sheep. Protein appeared to be unavailable to animals fed leaves stored for 24 months or longer. The effect of storage on organic matter digestibility was less dramatic and due largely to the loss of soluble cell contents ($r = 0.97$). As a result, the proportion of water-insoluble dry matter and lignin present in leaves increased with duration of storage while the proportion of water or acetone-water (60:40 v/v) soluble material decreased. Despite being unavailable in vivo, cellulase digestion released protein from the water-insoluble residues of stored leaves in greater amounts than that released from freshly-dried leaves. It appears likely that protein released from stored leaves was in the form of a complex and remained unavailable to the animal. Hydrolysable and condensed tannins were not detected in fresh or dried leaves and could not have acted as complexing agents. The seco-iridoid glycoside oleuropein was found in fresh tissue (69.9 g kg^{-1}) but concentrations decreased on storage in parallel with the observed decrease in crude protein digestibility ($r = 0.80$). (© Elsevier / Inra)

olive leaves / digestibility / sheep / protein / phenolic compounds

Résumé – Effets de différents modes de conservation des feuilles d'olivier sur leur valeur nutritive pour les ruminants. Des feuilles d'olivier (*Olea europaea* L) ont été stockeés dans différentes conditions durant des périodes allant jusqu'à 42 mois. La durée du stockage a eu d'importants effets sur la digestibilité chez le mouton des matières azotées des feuilles. Les matières

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azotées des feuilles stockées pendant 24 mois ou plus n´ont plus été utilisées par les animaux. Les effets du stockage sur la digestibilité de la matière organique ont été moins dramatiques et sont dus surtout à la perte du contenu cellulaire soluble (r = 0,97). Il en a résulté que la proportion de lignine et de matière séche insoluble dans l'eau présente dans les feuilles a augmenté avec la durée du stockage, tandis que le matériel soluble dans l'eau ou en mélange (60:40 v/v) acétone-eau a diminué. Les protéines des feuilles stockées, quoique non utilisables in vivo, ont été libérées des résidus insolubles dans l'eau par une cellulase en quantités plus grandes que celles qui ont été libérées des feuilles fraîches séchées. Il est probable que les protéines libérées par les feuilles stockées font partie d'un complexe inutilisable par l'animal. Les tanins hydrolysables et condensés n´ont pu être détectés dans les feuilles fraîches ou sèches et n'ont donc pas agi comme agent complexant. La glycoside seco-iridoïde oléuropéine a été trouvée dans les tissus frais séchés (69,9 g kg^{-1}) mais les concentrations ont diminué proportionellement avec le diminution de la digestibilité des protéines (r = 0,80) durant le stockage. (© Elsevier / Inra)

feuilles d'olivier / digestibilité / mouton / protéine/ composés phénoliques

1. INTRODUCTION

The use of tree foliage as feed in animal production systems is a common practice in dry and semi-dry areas. In the Mediterranean basin, where approximately 98% of the world production of olives takes place [5], the most commonly used foliage comes from olive trees. Traditional management systems allowed goats and sheep to freely roam the olive groves feeding on the leaves from pruned branches. However, current practice favors gathering and burning prunings to limit the spread of parasites and to allow ploughing between trees. This has resulted in a shortage of ruminant feed in some parts of Spain and has renewed interest in developing alternative strategies for the utilization of olive by-products in animal feeding systems.

Any move to collect and store the prunings from olive trees involves drying the leaves. In a preliminary study, Gómez-Cabrera et al. [8] found that the dry matter digestibility and the availability of protein to ruminants fell substantially when olive leaves were air-dried at ambient temperatures. The extent of these losses was determined by the severity and duration of the drying. This was contrary to observations made with the tannin-rich oak leaves where drying appeared to have no or even a slightly beneficial effect on nutritive value [15, 16].

It is well recognized that if temperatures exceed about 50 °C during the drying of feed samples the apparent lignin and fiber contents increase [27], additional nitrogen becomes bound to the fiber fraction [6, 7, 24] and digestibility may be reduced [13]. However, the proximate analysis of oven-dried and air-dried olive leaves by Gómez-Cabrera et al. [8] provided little evidence of such changes and an alternative mechanism responsible for the observed substantial fall in crude protein digestibility was required. This led Gómez-Cabrera and his colleagues to suggest that phenolic compounds in slowly dried leaves may affect palatability and inhibit the digestion process. The aim of the present paper was to examine the effect of a range of drying processes, differing in duration, temperature and exposure to moisture, on nutritive value and to establish whether the many phenolic compounds present in olive leaves (see *table I*; [12]) are implicated in the reduced organic matter and protein digestibility of the dried leaves when fed to sheep.

2. MATERIALS AND METHODS

2.1. Collection and storage of olive leaves

Collections of mature branches were made during routine pruning of Picual and Hojiblanca cultivars of olive trees (*Olea europaea* L.) in February in 1987 and again in 1990 and 1991. Fresh leaves were collected from the same trees in September 1992. Branches acquired in 1987 were either immediately dried in a forced-air oven at 60–65 °C for 16 h and the leaves then separated from branches by hand (OD sample) or they were left to air dry in a shed at ambient temperature as previously described by Gómez-Cabrera et al. [8]. After 3 months the leaves were removed from air-dried branches by crushing and manual separation. Samples of the leaves were then stored in sealed containers until required (sample AD3) or left under ambient conditions for a further 21 (sample AD24) or 39 months (sample AD42). Branches cut from trees in 1990 were either baled ($0.6 \times 0.4 \times 0.3$ m) and left to dry in a shed at ambient temperature for 9 months after which time the leaves were manually removed (BB sample), or the leaves and twigs, removed mechanically from the branches immediately after pruning, were left unbaled to air-dry and after 24 months (BP24) and 42 months (MP42) the leaves mechanically separated from the twigs. Finally, a sample of branches harvested in 1991 was allowed to become saturated with rain water before storing under dry conditions in a shed for a period of 12 months followed by the mechanical removal of the leaves. Occurrence of fermentation was evident in this sample (BF). Separated leaf material from all samples was stored without further treatment for digestibility determinations or ground to pass a 1 mm screen in preparation for analysis.

Two samples, sufficient for analysis only, were prepared from the fresh leaves collected in September 1992: one freeze-dried to produce minimum change on drying (sample FD) and the second, comparable to the OD sample prepared for digestibility experiments, dried in a vacuum oven at the lower temperature of 40 °C for 144 h to minimize oxidative damage (sample VO).

2.2. Digestibility determinations

The digestibility of the eight samples of olive leaves prepared after various conditions of storage were measured using four adult Segureña sheep as described by Gómez-Cabrera et al. [8]. The amount of olive leaf offered to the sheep was 70 g DM/kg$^{0.75}$ and 150 g of dehulled sunflower seed meal was mixed with the leaf as a protein supplement. The adaptation period was 14 days followed by 10 days of collection of feces. Dry matter, ash and crude protein content of leaves, diets and collected feces were determined following the recommended AOAC methods. Acid detergent fiber (ADF) and acid detergent lignin (ADL) were quantified by the method of Robertson and Van Soest [22]. Organic dry matter and crude protein digestibility of the olive leaves were calculated by difference, taking into account the measured digestibility of the sunflower component of the diet.

2.3. Extraction and analysis of leaf samples

Samples of leaf material (10 g) were extracted with petroleum ether to remove chlorophyll and lipids, followed by extraction either with water for 1 h at 40 °C or cold acetone-water (60:40 v/v) overnight. Petroleum-ether extracts were discarded but the acetone-water and aqueous extracts were retained, freeze-dried (after removal of any acetone), and weighed. Freeze-dried extracts and extracted residues were freezer-milled under liquid nitrogen for 3 min (Spex 6700 freezer-mill, Spex Industries Inc., Edison, NJ, USA) prior to analysis.

Total phenolics were measured using the acetyl bromide method [17, 18] and expressed as ferulic acid equivalents by reference to a standard calibration curve. Extracts were examined for the presence of hydrolysable tannins (gallotannins) by the method of Inonue and Hagerman [10] and condensed tannins using the method described for proanthocyanidins by Porter et al. [21]. Oleuropein was extracted from olive leaves (20 mg) by sonication with 80% ethanol (1 mL) containing 0.01 mg anisic acid as internal standard. The sample was centrifuged, the residue removed and water (1 mL) added to the supernatant prior to removal of the ethanol under a stream of nitrogen. The

sample was then applied to a C18 solid phase extraction cartridge (Accubond, J.W. Scientific, Folsom, USA), which had been pre-equilibrated with methanol (3 × 1 mL) and water (3 × 1 mL), washed with water (1 mL) and eluted with 50% ethanol (2 × 400 μL). The ethanol was partially removed under a stream of nitrogen and 20 μL of the predominately aqueous sample analyzed by HPLC using a 100 × 4.6 mm 5 μ Spherisorb ODS2 (Hichrom Ltd., UK) column. The eluent was 35% methanol in 0.5% acetic acid at a flow rate of 1 mL min^{-1} and detection was by UV at 280 nm.

The protein content of the extracts was determined by the protein-dye binding method [1] using bovine serum albumin as standard, or as crude protein (N × 6.25) calculated from the nitrogen content determined by Kjeldahl digestion. Crude protein in the insoluble residues was calculated from Kjeldahl nitrogen determinations. Water or water-acetone-insoluble residues were incubated with a commercial cellulase preparation (Fluka ex *Trichoderma reseii*) for 24 h at 40 °C in 20 mM acetate buffer (pH 4.5), and the dry matter and nitrogen content of non-digested material measured and the values obtained used to calculate the extent of cell wall breakdown and protein (nitrogen) release.

The results were subjected to statistical analysis using the PROC MEANS and PROC CORR procedures of the SAS software package [23].

3. RESULTS

3.1. In vivo degradability of stored leaves

Increasing the severity or duration of the drying conditions had a marked effect on both organic matter (OMD) and crude protein digestibility (CPD) in sheep (*table I*). Comparison of the OD and AD3 samples, which had similar OMD values but different CPD, suggested protein digestibility was more sensitive to heat than to duration of storage but comparison of AD3, AD24 and AD42 showed that both fractions were affected by storage. Since the proportion of ADF and ADL present in the leaf samples increased with storage it is probable that any decrease in OMD was largely a reflection of a loss initially of highly degradable cell contents

Table I. Dry matter (DM), acid detergent fibre (ADF), acid detergent lignin (ADL) and crude protein (CP) content (g kg^{-1} leaf dry matter) and organic matter (OMD) and crude protein (CPD) digestibility (%) of olive leaf samples dried under various conditions. Values for the OD and AD3 samples have been published previously by Gómez-Cabrera et al. [8] and are given here for completeness of data.

Treatment	DM	ADF	ADL	CP	OMD[1]	CPD[1]
OD	956	264	168	176	50.6^{ef}	13.0^{b}
AD3	942	281	150	151	53.6^{f}	23.6^{c}
AD24	936	345	233	204	43.1^{d}	9.9^{b}
AD42	921	334	192	181	43.1^{d}	14.0^{b}
BB	941	255	163	157	44.8^{de}	7.6^{b}
MP24	894	425	249	153	31.6^{c}	-6.0^{a}
MP42	906	441	266	169	23.4^{b}	-14.0^{a}
MF	864	537	305	166	17.7^{a}	-4.0^{a}
FD	971	266	155	146	n.d.	n.d.
VO	960	313	205	139	n.d.	n.d.
S.E.					22	23

n.d., not determined.

[1]Mean separation by Tukey's test, $P \leq 0.05$. Values with the same superscript are not significantly different.

and latterly of the breakdown of the more digestible cell wall material. The solubility of DM in acetone-water, shown in *table II*, was consistent with this view and a direct relationship (r = 0.95) was found between soluble dry matter and the OMD determined *in vivo* (*figure 1*). Water alone was universally less effective than acetone-water in solubilising both dry matter and protein (data not presented) although there was still a direct relationship between OMD and soluble dry matter (r = 0.97).

The amount of protein extracted by water alone was half or less than the amount extracted by acetone-water from the corresponding sample shown in *table II*, although the magnitude of the difference did not obviously relate to treatment. Direct measurement of soluble protein by a dye-binding method and estimates made from the nitrogen content (crude protein) were in good agreement for the freeze-dried and vacuum oven dried samples but differed in those samples exposed to head or prolonged storage. In these samples the dye-binding method gave lower values than the crude protein determination, possibly implying some complexing of the protein. While the total crude protein content varied depending on dry matter content (*table I*), the proportion of total protein represented by the acetone-water insoluble fraction remained essentially constant (0.80–0.84) in all but one sample. The exception was sample MF which had been deliberately encouraged to ferment on storage. In this sample, the amount of soluble protein was substantially higher and the insoluble protein fraction was (0.70) correspondingly reduced.

The observed solubility of protein in acetone-water or water alone suggested that, even if none of the insoluble protein was potentially degradable, 15–20% of the total crude protein should be available to the animal and that in the MF fraction this value would be closer to 30%. However, this was not supported by the animal experiments in which few of the in vivo CPD values approached this figure and in the case of the MP24, MP42 and MF samples, none of the protein apparently was available (*table I*). In apparent contradiction to the in vivo results, in vitro digestion of the water- or acetone-water-insoluble residues of the leaves with a commercial cellulase resulted in substan-

Table II. Amount of dry matter (DM) and protein soluble or insoluble in acetone-water in olive leaves subjected to various methods of storage. Soluble protein was determined directly as protein by a dye-binding method [1] (B) or calculated from Kjeldahl nitrogen values (K). Values are the means of duplicate determinations.

Treatment	Leaf dry matter ($g\ kg^{-1}$)				
	Insoluble DM	Insoluble protein (K)	Soluble DM	Soluble protein (B)	Soluble protein (K)
OD	589	141	393	22	35
AD3	632	126	362	18	25
AD24	676	166	326	26	38
AD42	677	149	323	22	32
BB	603	133	381	17	24
MP24	749	124	239	36	29
MP42	772	132	228	33	37
MF	814	116	184	68	50
FD	602	122	394	24	24
VO	569	120	418	19	19

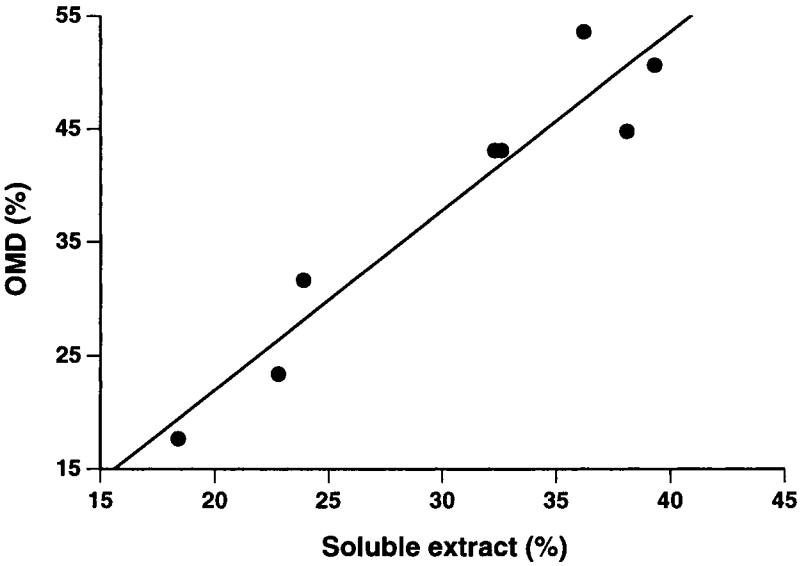

Figure 1. The relationship between organic matter digestibility (OMD) of olive leaves determined in vivo after various storage treatments and the percentage of dry matter soluble in acetone-water (OMD = –9.67 + 1.58 soluble extract, r = 0.95, S.E. = 4.1).

tial solubilisation of both dry matter and crude protein (*table III*, results for water-soluble extracts only shown). The crude protein released from stored leaves was comparable with or greater in amount than that released from the oven (OD) and freeze-dried (FD) samples prepared soon after harvest in the laboratory. Extended storage (24 and 42 months) and fermentation (MF sample) appeared to both conserve and promote the dry matter and crude protein in vitro degradation of the samples in marked contrast to the results observed in vivo.

3.2. Anti-nutritional factors in olive leaves

Since cell wall degrading enzymes were able to digest a substantial proportion of all leaf samples in vitro, the poor digestibility of dry matter and protein observed for some samples when fed to animals related either to the biological unavailability of the released material or to the direct inhibition of the rumen microflora. Hydrolysable or condensed tannins were not detected in any water or acetone-water extracts of olive leaves and were discounted as a possible anti-nutritional factor. The seco-iridoid glycoside oleuropein was found in substantial amounts (*table IV*) in freeze-dried samples but in lower concentrations in leaves which had been stored. The extent of oleuropein disappearance was related to the fall in OMD (r = 0.83) and to the reduction in CPD (r = 0.80).

The acetyl-bromide method provided an alternative and more specific measure of lignin content of the water and acetone-water insoluble residues than did ADL although there was a clear relationship

Table III. Total water-insoluble dry matter (DM) and crude protein (CP) content and amount of DM and CP solubilised by the action of a commercial cellulase enzyme preparation from olive leaves subjected to various storage treatments. Values are the means of duplicate determinations.

Treatment	Water-insoluble dry matter (g kg^{-1})	DM released from insoluble fraction by enzyme (g kg^{-1}) (% of total)	Water-insoluble crude protein (g kg^{-1})	CP released from insoluble fraction by enzyme (g kg^{-1}) (% of total)
OD	707	490 (69.3)	126	83 (66.0)
AD3	721	454 (63.0)	116	72 (61.8)
AD24	781	582 (74.5)	149	106 (71.6)
AD42	794	566 (71.3)	126	87 (69.2)
BB	751	478 (63.3)	125	81 (65.1)
MP24	865	656 (75.9)	112	85 (75.9)
MP42	873	694 (79.9)	115	92 (79.8)
MF	902	723 (80.2)	109	88 (80.8)
FD	714	535 (75.0)	106	76 (71.7)
VO	732	513 (70.1)	107	67 (62.6)

Table IV. Oleuropein content of olive leaves subjected to various storage conditions and the total phenolic (TP) content of their acetone-water extracted residues expressed as a percentage of the residue and original leaf dry matter (DM). Values are the means of duplicate determinations.

Treatment	Oleuropein content of leaf DM (g kg^{-1})	TP content of acetone-water insoluble extract of leaves (g kg^{-1})	TP content of acetone-water insoluble extract expressed as original leaf DM (g kg^{-1})
OD	36.0	34.3	20.2
AD3	29.2	39.7	25.1
AD24	6.6	37.5	25.4
AD42	10.7	43.9	29.7
BB	12.3	54.8	41.0
MP24	1.4	58.9	45.5
MP42	1.1	63.7	51.9
MF	0	n.d.	n.d.
FD	33.6	25.9	14.8
VO	69.9	25.0	14.2

n.d., not determined.

($r = 0.79$) between the two measures. As would be expected, there was a strong negative relationship between the phenolic content of the insoluble residues and amount of dry matter extracted by water ($r = -0.96$) or acetone-water ($r = -0.99$), mirroring the relationship with ADL. A strong negative relationship ($r = -0.96$) was found between the phenolic content of extracted leaf residues and OMD (*figure 2*) but the relationship with CPD was considerably weaker and not significant ($r = -0.75$).

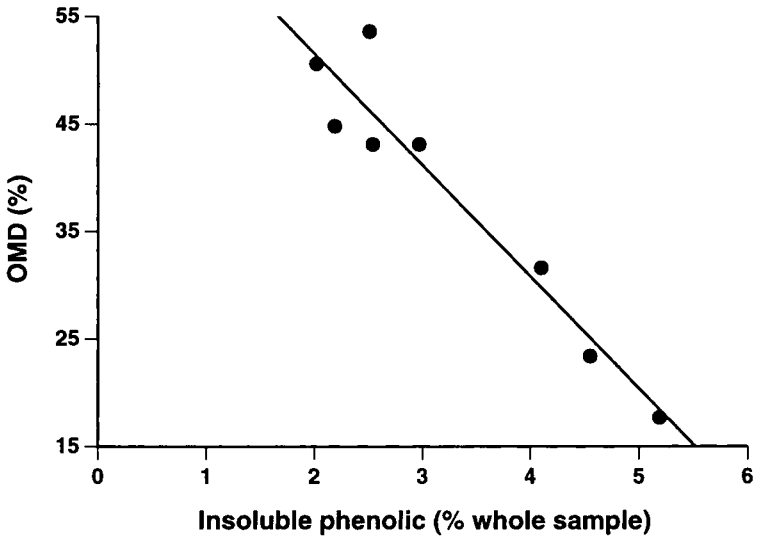

Figure 2. Relationship between organic matter digestibility (OMD) and the contribution made by acetone-water insoluble total phenolics to total dry matter in olive leaves subjected to various storage treatments (OMD = 72.29 – 10.4 insoluble phenolic, r = –0.96, S.E. = 3.97).

4. DISCUSSION

Tree leaves used as an additional or alternative source of nutrients in many tropical countries often contain substantial amounts of condensed tannins. These have been shown to depress performance when fed as a high proportion of the total diet to ruminant animals. Drying of such leaves, whether in the field or in the laboratory does not appear to greatly change their tannin content or nutritional value [4, 13, 14–16]. Where there is an effect of conservation, the tendency is to improve rather than decrease nutritive value of tannin-rich leaves [16, 19].

The olive leaves used in this study were shown to be free of hydrolysable and condensed tannins. Their response to drying more closely resembled the forage samples studied by Mahyuddin et al. [13]. In particular, crude protein digestibility was found to be zero in three of the conserved samples and much reduced in others, implying the formation of a more stable complex than is typical of tannin-protein interactions [20] with no post-ruminal release of protein. Coffey et al. [3] working with fescue, Scehovic [24] with a range of forages and Gómez-Cabrera et al. [8] with olive leaves have all reported that forage drying causes a greater binding of nitrogen to the fiber fraction.

Much of the nitrogen present in water- and solvent-extracted olive leaves could be released by the action of a commercial preparation of cell wall degrading enzymes. Since a similar release of protein would be expected within the rumen, the resulting lack of availability to the animal indicates that the nitrogen, although partially solubilised, remains complexed in a form resistant to host or microbial proteolytic attack. In the absence of condensed tannins, the most likely candidates for complex formation with protein are other phenolic compounds. It has long been recognized that virtually all com-

pounds with a phenolic functionality can covalently link to sulphur-containing and other amino acids, a process aided by moisture, heat and time [25]. The ability of acetone-water to extract more nitrogen from stored samples than water alone and the lower soluble protein estimates provided by the dye-binding also are consistent with some complexing with a phenolic fraction.

In addition to lignin in the cell wall fraction, olive leaves and fruit are characterized by a high concentration of oleuropein, a seco-iridoid glycoside which imparts the bitter taste to the fruit [11]. The concentration found in the FD leaves (70 g kg^{-1} dry matter) fell within the range normally encountered, which is highly variable and can reach 90 g kg^{-1} dry matter [26]. There was a considerable fall in oleuropein content of leaves with storage, the extent of which could be correlated with the loss of dry matter and CPD. However, the two may not be causally related. Loss of extractable oleuropein can occur because of hydrolytic breakdown [2, 28] or slow oxidative polymerization [9] without any interaction with protein. Polymerization of oleuropein or its breakdown products would decrease solubility and make it difficult to distinguish from the lignin fraction in any gravimetric assays. Further work would be needed to establish whether the protein which apparently becomes complexed with phenolic material is associated primarily with lignin, oleuropein, or both. However, any suggestion that oleuropein might be toxic to the rumen microflora can be discounted. The highest digestibilities were recorded for the samples with the greatest oleuropein content.

OMD was evidently more sensitive to the duration of storage than to heat. Water and solvent-soluble dry matter decreased with storage and the amount of reduction was strongly related to the decrease in OMD. There was a concomitant rise in cell wall content, particularly for the more lignified cell types, as shown by the increase in lignin, whether measured as ADL or by the acetyl bromide method. However, as indicated previously, not all of the proportional increase in lignin may be due to the loss of cell contents. Complexation with phenolics may also have contributed material which was detected as ADL. Overall, this pattern of events is consistent with the decrease in OMD due principally to loss of cell contents through leaching or microbial action during the drying or normal senescence processes and possible with the loss of more readily degradable cell wall material. Thus OMD was principally determined by the amount of material which was insoluble in water or solvent and its apparent lignin content.

It was suggested previously that removal of leaves from twigs and branches had a negative effect on both OMD and CPD [8]. Although removal of leaves might, in the short term, promote autolysis and loss of cell content, there is little to suggest that this is significant in the longer term. The prime factor in reducing nutritive value appears to be duration of storage, followed by heating or fermentation when leaves were exposed to rainfall. This argues for leaves to be collected dry, stored under cover and consumed within a 3–4 month period.

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