# Measurement of Cell Rupture in Macerated Forages

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A method of measuring cell disruption in ground leafy plants using chlorophyll (Chl) analysis is presented. The Chl method is positively correlated ($r \geq 0.95$) with a cell rupture procedure based on protein nitrogen analyses. Grinding tests with alfalfa demonstrated the method could be used to detect changes in the amount of cell rupture induced by altering grinder parameters such as plate gap setting and feed rate. The usefulness of the method was further demonstrated by its application to the optimization of some operating parameters for a commercial-size hammer mill.

Commercial leaf protein plants are currently established in Europe and the United States (Edwards et al., 1981) where they operate as adjuncts to alfalfa dehydration plants. The incoming alfalfa is wet fractionated by grinding the fresh chops and expressing the protein rich juice, separating it from the fiber. The heat-coagulated and air-dried whole leaf protein concentrate (LPC) is utilized as an ingredient in swine (Cheeke et al., 1977) and poultry (Kuzmicky and Kohler, 1977) rations, mainly for its vitamin, protein, and pigment content. The fiber fraction, which retains protein in excess of that required for ruminants, is dried and pelleted for ruminant rations.

A quantitative estimate of cell rupture was needed to evaluate and optimize process parameters during the grinding and pressing operations, which have the greatest impact on the yield of LPC. The literature revealed several methods used to estimate cell rupture, including microscopic examination of plant residues (Wildman and Bonner, 1957), measurement of the moisture reduction in plant materials (Koegel et al., 1973; Basken et al., 1975), conductivity ratios (Emetarom and Barrington, 1977), measurement of the protein released from crushed leaves (Addy et al., 1975; Anaya-Serrano, 1978; Carroad et al., 1981), and chlorophyll assay (Miller et al., 1979a; Lees et al., 1981).

This paper presents a method of determining cell rupture in ground forage samples by measuring the ratio of the chlorophyll (Chl) released from broken cells to the total Chl. The strong chlorophyll absorbance peak at 663 nm is used as a measure of the amount of Chl in solution, and since only a ratio between similar samples is required, it is not necessary to quantitatively determine the chlorophylls. Results are compared with a protein method for measuring degree of cell rupture (DCR). Experiments were also conducted to demonstrate that the new method is sensitive enough to detect DCR changes resulting from changes in process parameters.

## EXPERIMENTAL SECTION

**Raw Materials.** Alfalfa (*Medicago sativa*) samples (7–10 kg) were hand cut and immediately (~15 min) brought to our laboratory where they were chopped into 2–5-cm lengths with a paper cutter and mixed well in a large plastic bag before grinding. Larger quantities of alfalfa were mechanically harvested, chopped, and trucked to our pilot plant in Berkeley from a commercially grown field near Davis, CA.

**Equipment.** One-kilogram samples for evaluation of the DCR method were ground with a 20.3-cm (8 in) single-disk attrition mill (C. E. Bauer, Springfield, OH, style 148–8) (Miller et al., 1979b) by using a pair of fine ribbed plates (no. 8118). The mill was powered by a 5-hp motor and turned at 3600 rpm. The distance between the plates was varied from 0.051 mm (0.002 in.) to 1.52 mm (0.060 in.) to produce samples with different amounts of cell rupture.

Samples of ground forage were also collected at several feed rates from the discharge of a 101 cm (40 in.) vertical hammer mill (Master Crusher, Owens Manufacturing Co., Verndon, NE) previously described (Edwards et al., 1978; Carroad et al., 1981). Grinder energy consumption was measured by using a Watt/Watthour transducer (Columbus Scientific Model XL-6070, Columbus, OH). Grinder feed rates were determined by weighing all material collected from the grinder exit during a timed interval.

All samples were stored in sealed plastic bags on ice in the dark (Bruinsma, 1963; Strain and Svec, 1966) until they were processed, usually within 3 h. Our experience showed that ground alfalfa lost about 3% Chl/h at 22 °C but less than 1%/h at 4 °C as indicated by decreased absorbance readings.

**Analytical Methods.** The percent total solids of fresh samples (80–100 g) was calculated from the loss in weight after drying in a forced draft oven for 4 h at 105 °C. Freeze-dried samples were ground through a 1 mm mesh screen before being analyzed for nitrogen, crude fiber (CF), and total solids (TS) by standard methods (AOAC, 1975). All nitrogen determinations were by the Kjeldahl method (AOAC, 1975).

A Cary Model 15 spectrophotometer with 1-cm quartz cells and Teflon stoppers was used to determine the absorbance at 663 nm of Chl samples. The reference solution was 87% acetone by volume (85 + 15) and the base line was adjusted to zero at 720 nm.

**Chlorophyll DCR Method.** *Liberated Chlorophyll (Chlorophyll Extracted from Broken Cells).* Under subdued light, a selected sample was thoroughly hand mixed, carefully subdividing agglomerated lumps and pieces. An 80.0-g portion was weighed into a 2-L Griffen beaker to which 800.0 g of an aqueous extraction solution [0.5% sodium dodecyl sulfate (SDS), 1.0% 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), adjusted to pH 7.5 with 3 N HCl] was then added. The mixture was magnetically stirred in a darkened room (~27 lx), maintaining continuous circulation of the extraction solution without forming a vortex. Stirring was stopped after 40 min and a 25 mm diameter by 15 cm glass tube, with a nylon fine-mesh screen (~0.1-mm openings) covering the bottom, was lowered about 8 cm into the 2-L beaker. Two 5-mL aliquots of screened juice from within the glass tube were each pipetted into 25-mL volumetric flasks containing 20 mL of reagent-grade acetone. These samples were stored in the dark for a minimum of 10 min but usually overnight. Samples were adjusted to 25-mL total volume with acetone and filtered through fluted filter paper (E & D no. 513) prior to determining the absorbance. In some experiments,

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additional aliquots were removed for nitrogen analyses.

*Total Chlorophyll (in Raw Material).* A 30.00-g sample was weighed into a 250-mL screw-cap jar, enough acetone was added to make an 87% aqueous solution (~136 mL for a sample with 80% moisture), and the tightly sealed jar was stored in a refrigerator until the extraction procedure could be completed, usually the following day. In a darkened room, the sample was transferred to a 1-qt glass Waring blender bowl equipped with a splash guard and cover. The blender speed, controlled with an autotransformer, was gradually increased to 13000 rpm (low speed) to prevent excessive splashing. The blender was stopped after 1 min, and fiber from the bowl sides and splash guard was recombined with the acetone in the bowl bottom. After being blended for another minute, the entire contents of the blender bowl was transferred to a 350-mL sintered glass funnel (ASTM 40-60) mounted on a 1 L, aluminum foil wrapped, vacuum filter flask attached to a water aspirator. After the Chl solution was drawn through the filter, the fiber mat was rinsed with 80–100 mL of 87% acetone, which was combined with the original Chl solution. The fiber was transferred to the blender bowl and, with an additional 100 mL of 87% acetone, was blended for 1 min and returned to the sintered glass filter. The fiber mat was rinsed to remove any residual Chl. The filtrate was transferred to a 500-mL volumetric flask and stored in a cool dark place. The filtrate was adjusted to 500 mL and aliquots were removed for absorbance readings. Acetone extracts of alfalfa usually required a 3:10 dilution to reduce the absorbance readings to an acceptable level.

**Protein DCR Method.** The protein method of determining the degree of cell rupture (DCR) previously described by Miller et al. (1979a) and Carroad et al. (1981) was modified and used for comparison with the Chl DCR method. The protein nitrogen (PN) released by cell rupture was determined on duplicate 20-g aliquots of the liberated Chl extract taken immediately after the samples for Chl analysis.

Non-protein nitrogen (NPN), defined as the nitrogen soluble in 10% trichloroacetic acid (TCA) solution, was determined by extracting a 1-g freeze-dried sample overnight with 40 mL of 10% TCA and analyzing a 30-mL aliquot for nitrogen.

**Calculations.** The Chl DCR is defined (eq 1) as the

$$\mathrm{DCR} = E/T \qquad (1)$$

ratio of the amount of Chl released from ruptured cells ($E$) to the total amount available ($T$). Since the absorbance of Chl in solution is proportional to its concentration, we can obtain eq 2 and 3 from Beer's law. AbsE = absor-

$$E = (\mathrm{Abs}E)[\mathrm{ES} + (\mathrm{FW}\text{-}\mathrm{DW}_1)(\mathrm{DCR})]/[K_1(\mathrm{DW}_1)D] \qquad (2)$$

bance of extraction solution, ES = weight of extraction solution, FW = weight of fresh sample, $\mathrm{DW}_1$ = weight of dry sample (FW × fractional dry matter), $K_1$ = dilution factor, and $D$ = density of extraction solution. AbsT =

$$T = (\mathrm{AbsT})(V)/[K_2(\mathrm{DW}_2)] \qquad (3)$$

absorbance of acetone extract, $V$ = volume of acetone extract, $K_2$ = dilution factor, and $\mathrm{DW}_2$ = weight of dry sample.

The weight of the extraction solution in eq 2 is adjusted to correct for added liquid from ruptured cells by using the DCR value as an approximation of the fraction of the total liquid released by grinding. The DCR is assumed to be 1 for the first calculation of $E$. The new value for DCR is obtained by dividing eq 2 by eq 3 and it is then used to recalculate $E$. The DCR value converges rapidly so that no significant change occurs after four iterations. The magnitude of the correction will depend on the dry matter and degree of cell rupture but is about 3–4% for samples containing 20% dry matter and 60% cell rupture.

Equation 3 is used to calculate the term for the total amount of Chl present in the raw material ($T$).

**Protein Degree of Cell Rupture.** The protein degree of cell rupture is the ratio of the true protein nitrogen liberated from broken cells to the total amount of true protein nitrogen in the raw material, reduced to a common basis (eq 4). The true protein nitrogen content of the raw material is taken to be the total nitrogen content minus the nitrogen soluble in the TCA solution (eq 5). PN =

$$\mathrm{DCR} = \mathrm{PN}/(\mathrm{TN} - \mathrm{NPN}) \qquad (4)$$

protein nitrogen from extraction solution (g/g of dry sample), NPN = nonprotein nitrogen (g/g of dry sample), and TN = total nitrogen (g/g of dry sample). $N$ =

$$\mathrm{PN} = N[\mathrm{ES} + (\mathrm{FW} - \mathrm{DW}_1)(\mathrm{DCR})]/[(\mathrm{DW}_1)V_a D] \qquad (5)$$

TCA-precipitable nitrogen (g) and $V_a$ = volume of extraction solution analyzed.

## RESULTS AND DISCUSSION

**Chlorphyll DCR Method.** A preliminary experiment to determine a suitable sample size indicated that 80.0 g of ground material would yield the desired precision. A standard deviation of 1.3% was obtained in an experiment with 10 subsamples of ground alfalfa extracted with an aqueous solution. A similar experiment with acetone as the extractant resulted in a standard deviation of 1.0% for 30.00-g alfalfa samples. Larger samples could reduce the error but would be more cumbersome to process and require more solvent.

Although water was used as an extraction solvent in DCR methods utilizing conductivity ratios (Emetarom and Barrington, 1977) and protein determinations (Anaya-Serrano, 1978), it is unsatisfactory for Chl extraction because Chl is insoluble in water. Also, the chloroplasts are only slowly and incompletely removed with distilled water and will agglomerate and precipitate. The slow increase in Chl absorbance with increasing stirring time for samples extracted with distilled water can be seen in Figure 1. Detergent solutions have been used to solubilize Chl and chloroplast proteins during the fractionation of chloroplasts (Shibata, 1971) and the characterization of Chl–protein complexes by electrophoresis (Machold and Meister, 1979). Preliminary experiments with Tween-60, a non-ionic emulsifier (sorbitan monostearate), and sodium dodecyl sulfate (SDS), an amphiphilic detergent, showed significant improvements in the extraction of Chl over that of distilled water used as a control, with SDS being the most effective. The data presented in Figure 1A shows that although SDS improved the initial extraction of Chl, the absorbance declined rapidly with stirring time. Initially the SDS solution was at pH 7.3, but it had decreased to pH 6.0 after 2.5 h of stirring. This decrease in pH may explain some of the Chl absorbance loss, since Chl is rapidly converted into pheophytins and chlorophyllides at pH values below 7 and above 9. Additional experiments examining the effect of extraction solution pH on the absorbance of Chl verified that the maximum absorbance and stability were obtained above pH 7.0. The addition of 0.1 M Tris, adjusted to pH 7.3, maintained the pH about 7.2 and prevented the rapid loss of Chl. Figure 1B,C show the effects of two concentrations each of SDS and Tris, all at pH 7.5, as a function of stirring time. In Figure 1B the doubling of SDS from 0.25% to 0.5% increased the maximum Chl absorbance 4–5%. Increasing the tris buffer from 1% (0.83

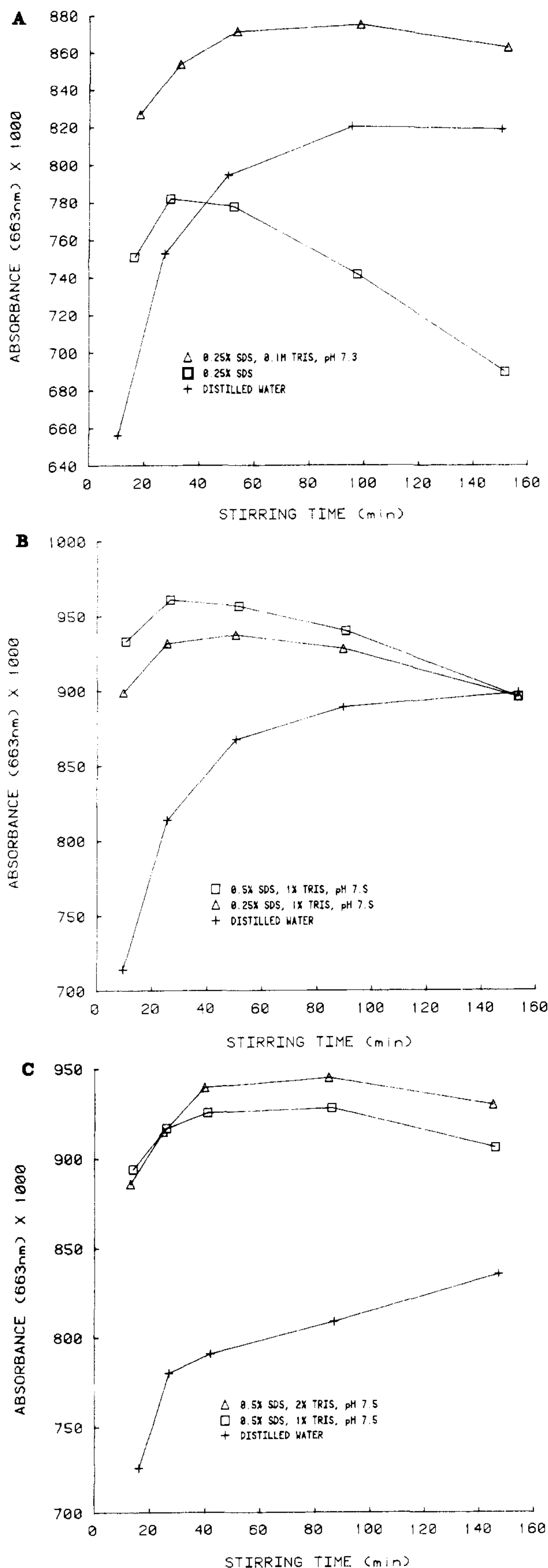


**Figure 1.** Chlorophyll absorbance of several extraction solutions as a function of stirring time. Three separate samples of ground alfalfa.

M) to 2% improved the Chl extraction about 2% (Figure 1C).

On the basis of the above data, the extraction solution contained 0.5% SDS to solubilize the Chl and 1.0% Tris buffer adjusted to pH 7.5 to reduce the rate of Chl degradation, and samples were stirred for 40 min before aliquot removal for absorbance readings. No Chl leached from fresh whole alfalfa leaves after soaking 6 h in the extraction solution, confirming that Chl was not being removed from unbroken cells.

The addition of $CaCO_3$, $MgCO_3$, or $Na_2CO_3$ to plant material with acid cell sap is recommended to prevent the degradation of Chl during the extraction process (AOAC, 1975; Strain et al., 1971; Willstatter and Stoll, 1928). Since the acetone-extracted sample was not buffered, $CaCO_3$ was added to a sample of alfalfa before extraction to determine if the addition of a basic salt would help preserve the Chl during extraction. No improvement in extraction or stability of Chl was observed. It should be noted that alfalfa does not have a highly acid cell sap; the fresh juice usually has a pH of 5.8 to 6.2, depending on the elapsed time since harvesting, the storage temperature, amount of tissue damage, and soil conditions.

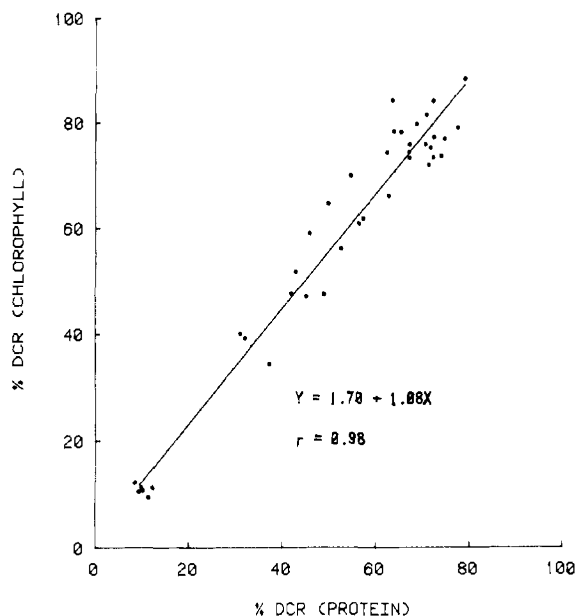


**Figure 2.** Scatter plot and least-squares regression equation of degree of cell rupture determined by chlorophyll assay vs. degree of cell rupture by nitrogen assay.

**Correlation with Protein DCR Method.** The DCR values obtained from ground alfalfa samples with the Chl and protein methods are shown in Figure 2. The data show that the cell rupture values obtained from nitrogen analyses and from Chl absorbance are highly positively correlated. A slightly higher DCR is obtained by the Chl method. This may be partially explained by the method of calculating the protein DCR values. It is assumed that all of the protein is available for extraction when a cell is ruptured but this is unlikely. Crook (1946) and Crook and Holden (1948) reported that on an average only about 75% of the total nitrogen could be removed from the leaves of a rather comprehensive list of plants. Dougall and Shimbayashi (1960) reported that from 25% to 40% of the nitrogen in tobacco callus tissue remained insoluble and bound to the cell wall after repeated extractions with phosphate buffer (pH 9.2) and 0.1 N NaOH. There is also evidence that protein tends to bind to the plant fiber when it is released from the cell and this could also cause a lowering of DCR values. A value for total protein nitrogen that is higher than the actual value will cause a low DCR value.

**Applicability of DCR Method to Processing Studies.** The Chl DCR method was tested by grinding samples

Table I. Experimental Data from Hammermill Grinding of Alfalfa[a]

| exp no. | agitation rate, rpm[b,e] | feed rate, kg/h | DCR, % | energy, kWh/1000 kg | DCR/$E$, %·1000 kg/kWh | DCR/$E$,[c] %·1000/kWh |
|---|---|---|---|---|---|---|
| 1 | 1630 | 2948 | 66.5 | 12.51 | 5.32 | 4.21 |
| | | 6033 | 65.6 | 7.03 | 9.33 | 6.93 |
| | | 6895 | 69.3 | 6.39 | 10.85 | 8.29 |
| | | 12927 | 61.5 | 4.61 | 13.34 | 8.49 |
| | | | 29.5[d] | | | |
| 2 | 1750 | 1470 | 84.1 | 26.36 | 3.19 | 4.44 |
| | | 3837 | 79.7 | 12.45 | 6.40 | 7.98 |
| | | 4504 | 78.0 | 12.26 | 6.36 | 7.48 |
| | | 7267 | 74.2 | 8.34 | 8.90 | 10.09 |
| | | | 9.6[d] | | | |

[a] Alfalfa total solids and percent fiber for experiments no. 1 and 2, respectively, were 24.8% and 18.94% and 25% and 21.99%. [b] Two sets of 3.2 mm thick, hardened hammers, 10-mm clearance between hammer tip and breaker bar. [c] Corrected to reflect only cell rupture and energy consumption during grinding. [d] Initial value for chopped alfalfa before grinding. [e] Unloaded grinder energy consumption was 10.97 kWh at 1630 rpm and 14.09 kWh at 1750 rpm.

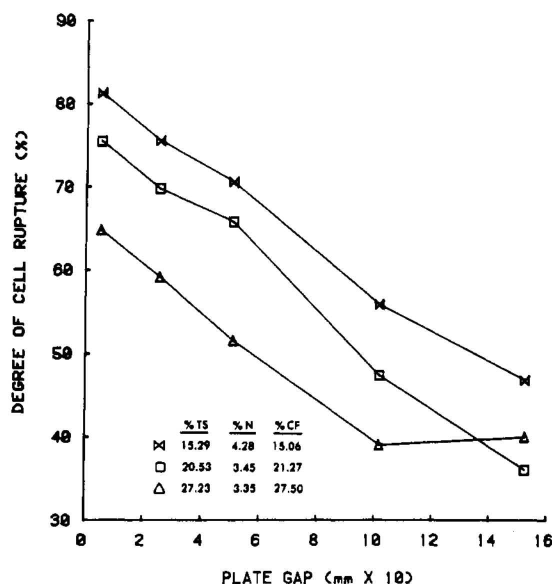


**Figure 3.** Cell rupture of alfalfa at three stages of growth, ground at several different attrition mill plate gap settings.

of alfalfa with a single-disk attrition mill. Results of a typical experiment are shown in Figure 3. In all cases, the expected inverse relationship between plate gap and the amount of cell rupture is clear. More cell rupture occurs with a narrow spacing between grinder plates than with a wider gap setting. The samples with higher crude fiber (CF) content have a low DCR. This relationship might also be expected, since the amount of fiber and cell lignification increase as plants mature, thus strengthening the cell walls and making them more difficult to rupture. The alfalfa samples show an increase in fiber content with stage of growth, and thus it appears that it is more difficult to achieve a given DCR as the plant matures.

The results of two additional experiments in which a hammer mill was used to grind alfalfa are summarized in Table I. In general, the grinding energy per 1000 kg of alfalfa and the percent cell rupture decreased while the amount of cell rupture per unit of energy increased with increasing feed rate. These results support those of Carroad et al. (1981).

Although direct comparison of the two experiments is complicated by the difference in the initial DCR of the two batches of chopped alfalfa, it is clear that the higher agitation rate produces more cell rupture. This agrees with observations reported by Addy et al. (1975) suggesting that impact systems for cell rupture would be most effective when operated at the highest feasible speed. More recently, a linear programming analysis of cell disruption variables reported by Carroad et al. (1981) indicated that the agitation rate should be maximized for optimum LPC production.

It is necessary to correct for the initial DCR of the alfalfa chops to get a valid comparison of the effect of the two agitation rates on the DCR per unit of grinder energy. The corrected data, calculated by subtracting the initial DCR of the chopped alfalfa and the no-load grinder energy, represent only the cell rupture that occurs during grinding. This correction results in an apparent lowering of the DCR/energy for the lower grinder agitation rate, indicating that energy is being expended without producing substantially more broken cells, possibly because the remaining unbroken cells tend to be the ones more resistant to disruption. Also, since there are more broken cells per mass of material, proportionally more energy is used in accelerating the material without rupturing additional cells. The DCR of the initial alfalfa, after chopping, in experiment 2 was only $^1/_3$ of that in experiment 1. Correcting the DCR per unit of grinder energy as in experiment 1 showed that substantially more cell disruption was occurring per unit of grinder energy at the higher agitation rate. Data such as this would aid in the selection of optimum operating conditions for maximizing LPC yields in a leaf protein plant.

In addition to the conclusions that can be drawn from the actual experiments, the results show that the DCR method can detect differences in DCR values that are due to changes in both process parameters and raw material quality. Since the Chl method is both easy and rapid, it should be extremely useful in process improvement and optimization studies.

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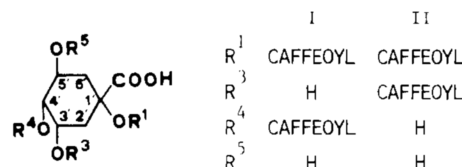
# Food-Related Applications of One- and Two-Dimensional High-Resolution Proton Nuclear Magnetic Resonance: Structure and Conformation of Cynarin

Ian Horman,* Raphael Badoud, and Willi Ammann

Cynarin, a depside isolated from artichokes, is shown to be 1′,3′-di-*O*-caffeoyl-D-(−)-quinic acid. This confirms one of the two structures suggested in earlier literature. The two caffeoyl residues are axially disposed on the cyclohexane ring of quinic acid. These results were obtained from 1 mg of cynarin in acetone solution and from only 90 μg in $D_2O$ solution, showing the capabilities of modern NMR techniques in providing extensive structural information on the small quantities of food constituents typically isolated by current high-performance separation techniques.

If the volatiles of foods have received much attention during the past decade, the same cannot be said for food solids. For these, a major barrier has been the availability of methods capable of providing comprehensive structural and conformational information on the small quantities of substances typically isolated. This problem has been accentuated by the development of separation techniques like high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) that permit isolation of constituents in a highly pure state but often in submilligram amounts. Recent advances in one-dimensional (1-D) and two-dimensional (2-D) NMR methods have opened up new fields of exploitation of this technique, and we report here a study on cynarin using a 2-D homonuclear correlated (COSY) proton experiment along with the conventional 1-D spectrum, both recorded at 300 MHz. Cynarin provides a good example because it is of limited solubility, giving saturated solution at 20 °C in acetone-$d_6$ of 6.8 millimolar, and in $D_2O$ of 0.58 millimolar. The maximum quantities of sample analysed thus correspond to 1 mg in acetone-$d_6$ and 90 μg in $D_2O$.

Cynarin is a depside diester isolated from artichokes. It belongs to the chlorogenic acid family, which are shikimic acid metabolites in plants (Bu'Lock, 1965), and was first reported by Panizzi and Scarpati (1954) to 1′,4′-dicaffeoylquinic acid (I). Panizzi and Scarpati (1965) sub-




|  | I | II |
|---|---|---|
| $R^1$ | CAFFEOYL | CAFFEOYL |
| $R^3$ | H | CAFFEOYL |
| $R^4$ | CAFFEOYL | H |
| $R^5$ | H | H |

sequently published a revised structure, namely, 1′,3′-dicaffeoylquinic acid (II), based on oxidative cleavage, derivatization, and specific synthesis. Our results have confirmed structure II and have demonstrated the conformation of the quinic acid ring, which places the two bulky caffeoyl groups in axial positions.

It should be noted that throughout this article, numbering of the quinic acid ring follows IUPAC nomenclature recommendations and goes around the ring in the opposite direction to the original literature nomenclature: II was originally labeled 1,5-dicaffeoylquinic acid.

## EXPERIMENTAL SECTION

Cynarin, isolated from artichokes, was purchased from Roth Products (Basel, CH) and had mp 229 °C [cf. lit. mp 227–228 °C with decomposition, (Panizzi and Scarpati, 1954)]. It was used without further purification. Acetone-$d_6$ and $D_2O$ from Stohler Isotopes (Innerberg, CH) were at least 99.85% pure. Cynarin was dissolved to give a saturated solution at 20 °C either in acetone-$d_6$ (3.5 mg·mL$^{-1}$) or in $D_2O$ (300 μg·mL$^{-1}$), and a 0.3-mL solution in a 5 mm diameter sample tube was used for the NMR

Nestlé Research Laboratories, 1814 La Tour de Peilz, Switzerland (I.H. and R.B.), and Varian NMR Applications Laboratories, 6500 Zug, Switzerland (W.A.).

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