STUDIES ON SELECTED ALKALOIDS IN THE FESCUES OF INTEREST IN THE PRODUCTION OF NEW CULTIVARS

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Charles Ellington Ainsworth Carrington

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ABSTRACT

Carrington, Charles Ellington Ainsworth, M.Sc., The University of Manitoba, May 1980. Studies on Selected Alkaloids in the Fescues of Interest in the Production of New Cultivars.

Major Professor: Kenneth W. Clark, Department of Plant Science

Quantitative and qualitative methods of fescue alkaloid analysis were established. Thirty-four meadow fescue cultivars and two Festuca x Lolium hybrids were established in a completely randomized design on a clay soil in Winnipeg, Manitoba in the summer of 1978, and qualitatively screened for alkaloid content the same year. A comparison of the loline content observed with that of two samples determined by an independent researcher to be "low" loline percent and "high" loline percent, showed an intermediate level with no apparent variation in all plants checked. Perloline concentration varied from "zero" to "very high". Perlolidine was not identified. The same plants were quantitatively screened for perloline content in the summer of 1979 on a regrowth basis. Perloline levels ranged from 0-6051.2 µg/gm dry matter. There were no significant variations in perloline content of plants within a cultivar; however, there was a significant variation in perloline concentration in plants between cultivars.

INTRODUCTION

Meadow fescue (Festuca elatior L.) and tall fescue (Festuca arundinacea Schreb.) are forage grasses with considerable potential for high productivity. Both grasses have similar morphological characteristics and were introduced into North America by the early European settlers. Both species have produced luxuriant fall pastures in those areas to which they are adapted. Wheeler (1950) suggests that the potential of meadow fescue for forage has not been fully appreciated, since it is capable of remaining green right up to the time that the ground freezes during winter. Bush and Buckner (1973) suggest that the chemical composition of tall fescue is equal to that of other forage grasses in areas where it is adapted.

For soils in Manitoba with ample moisture and good drainage, meadow fescue is recommended for pasture and seed production. Moreover, meadow fescue was a component of the most recommended hay and pasture mixtures in Manitoba since it is productive with alfalfa after bromegrass has ceased growth. But, in the mid 1970's meadow fescue was deleted from the list of recommended mixtures in Manitoba. There are two possible reasons: a decline in the availability of seed and the unwillingness of many planters to accept the existing varieties. In Europe, the use of Canadian synthetic varieties of meadow fescue has also declined because of slower recovery and higher alkaloid levels relative to other established varieties.

Considerable work on fescue alkaloids has been done by researchers in the United States. This was initiated in 1944 as workers sought the cause of "fescue foot" in cattle grazing toxic tall fescue pastures. Though, probably not the cause of "fescue foot", these alkaloids have also been linked to some forms of fescue toxicity. Gentry (1969) found 11 alkaloids in fresh tall fescue grass; Yates (1963) found nine alkaloids in cured tall fescue hay. These findings have led to further research into factors affecting the concentration of alkaloid compounds, their inheritance and their relationship to the poor performance of animals grazing tall fescue pastures.

In fescues, perloline is thought to be the most important alkaloid. Others of physiological importance are loline and perlolidine. However, the concentration of the last two alkaloids necessary for any significant pharmacological activity is much greater than that for perloline. Bush et al (1970 and 1972) found that perloline had a marked inhibitory effect on in vitro digestion and they suggest that the primary site of action of perloline is on rumen bacteria. This, thus, resulted in a decrease in volatile fatty acid production.

This study at the University of Manitoba was intended to establish a quantitative method for the estimation of fescue alkaloids and to use this method to assess satisfactorily the potential of the fescues and fescue x ryegrass hybrids.

LITERATURE REVIEW

Description of Meadow Fescue and Related Species

The Fescues

Meadow fescue (Festuca elatior L.) and tall fescue (Festuca arundinacea Schreb.) were introduced into North America from Europe by the early settlers. Both grasses have similar morphological characteristics and as a result confusion existed as to the differences between them. Before their identification as separate species in 1950, tall fescue was usually regarded as a variety of meadow fescue and was called Festuca elatior var. arundinacea.

Meadow Fescue

Meadow fescue is a hardy short-lived grass attaining a height of 37.5 - 75 cm or even more on extremely fertile land. Although it prefers a heavy, moist soil, it performs well on light soils, or those with moderate water supply, if adequately fertilized and managed intensively. Wheeler (1950) suggests that for wet soils few grasses are better adapted. Meadow fescue does not propagate by root stocks; it does not produce a very heavy sod. Nevertheless, it has excellent seed producing characteristics.

In his research, Wheeler found that the species was first planted on a farm in Johnson County, Kansas. It has since been grown in areas of New England, North Atlantic and the Central States, and in the

Southern States on a smaller scale. Meadow fescue is of marked significance in Eastern Kansas, Nebraska, Missouri, Indiana and many agricultural regions of Western Canada; because of its ability to withstand more severe winter conditions, it is better suited than tall fescue.

Meadow fescue is also recognized in Western Europe as an excellent pasture grass. The chief factor which has so far limited its wide usage in Canada and the United States is its high susceptibility to leaf rust, Puccinia spp. In regions where it was established, its primary use was for seed production, which was sold in Europe.

Studies in the United States and Canada have shown that meadow fescue is a high yielding pasture species in those areas where it thrives best. Wheeler (1950) states that meadow fescue is especially suited for pasture because it is usually ready for grazing in early spring and continues growing late into the fall; however, it remains green until the ground is frozen. In Canada, meadow fescue is used primarily in combination with bromegrass and alfalfa. After bromegrass has produced a lush growth in spring and early summer, it rarely makes any significant fall growth. It is, therefore, during this period, with no competition and shading from bromegrass, that meadow fescue makes sufficient regrowth and provides the grass component in the mixture.

Tall Fescue

Tall fescue is a deep-rooted, long-lived perennial bunch grass with short underground stems. Its thick stands produce an even sod if kept mowed or grazed. It is well adapted to poor winter drainage; it has deep penetrating roots where soils are well drained. Cowan (1967) reported that in Klameth Falls, Oregon, a variety of tall fescue known as Alta has thrived on alkaline soil with pH of 9.5. Additional reports

by other researchers also cite instances where tall fescue is known to thrive on soils with a pH of 4.7. This adaptability of tall fescue to acid, alkaline and even saline soils has made its use widespread throughout the United States and Canada. Buckner and Cowan (1975) have found, however, that although tall fescue is widely adapted, it grows best in the transition zone separating the Northern and Southern regions of the United States; an area where most cool and warm season grasses are not well adapted.

Bush et al (1973) points out that seed production of tall fescue in the United States increased from 10,900 Kg in 1940 to 31.3 million Kg in 1970; suggesting a greater demand for this crop in the United States. Research by Templeton and Taylor (1966) show that yield from well-fertilized pure stands and tall fescue legume mixtures are approximately 7-9 tonnes/ha in the United States. The increased use of tall fescue has been attributed not only to the seeming resistance of tall fescue to rust, but also to its wide adaptation to various climatic and soil conditions along with other valuable agronomic qualities.

Chemical Composition

Bush and Buckner (1973) state that a comparison of tall fescue with other grasses grown in the South Eastern United States indicates that the chemical composition of tall fescue is equal to that of other forages.

Further research has shown that it is possible to alter significantly the chemical composition of fescue by management practices. Hojjati et al (1977) cite references by Jones (1974) in which it is suggested

that an increase in N fertilization usually causes an initial rise in protein levels, which decreases as the season advances. Fribourg et al (1978) found that N fertilizer not only increases N but also NO_3-N in plant tissue. Balasko (1977) suggests that reducing the amount of senesced tissue by increased N fertilization, will result in a reduction of leaching action of rain that would readily remove soluble sugars and fructosans from senesced forage, thereby retaining most of the soluble carbohydrate content.

Digestibility

Reports indicate that the digestibility of meadow and tall fescue is comparable to that of other forage grasses. Allinson (1971) has found that in controlled environmental chambers, long days and high temperatures, contributed to the lowering of in vitro cellulose digestibility of tall fescue plants harvested after 8 and 19 wks. Smith (1977) also cites literature which suggests that both long days and high temperatures significantly lowered the percent of in vitro dry matter digestibility (IVDMD). Balasko (1977) further suggests that by judicious use of fertilizer treatments and cutting management, quality tall fescue forage for winter grazing can be achieved.

The overall quality of forage has been found to be lower under conditions of excessive growth, whether this growth was a result of time of accumulation or of favourable environmental conditions. This has been attributed to a higher ratio of senesced to live tissues. Beaty et al (1978) found that green forage averaged 70.9% digestibility while that of dead forage averaged 42.4%, thus the amount of dead forage significantly altered feed quality. Watson et al (1978) state

that the digestibility of tall fescue exhibits seasonal variations with the lowest levels resulting from conditions of high temperatures and long days.

Description of Lolium and Lolium x Festuca Hybrids

Lolium

The ryegrasses (Lolium spp.) are considered to be bunch grasses. There are two main Lolium species used for forage in North America. These are perennial ryegrass and Italian or annual ryegrass. Perennial ryegrass (Lolium perenne L.) originated in Southern Europe, North Africa and Southeast Asia. It is believed that it was first cultivated for forage in England about 1677. It grows to a height of approximately 90 cm. The origin of Italian ryegrass (Lolium multiflorum Lam.) is uncertain. However, it was grown in the meadows of northern Italy in the 13th century. It reaches a height of 130 cm.

These ryegrasses are commonly used for hay and pasture in Australia, New Zealand, the British Isles and the temperate regions of Western Europe and the United States. Since they are not very winter hardy, they grow best west of the Sierra Nevada, the Cascade Range and in the southern humid areas of the United States (Frakes, 1973), but their use also extend northward along the Atlantic Coast. He concludes that Italian is distinguishable from perennial ryegrass by having attached awns, the annual or biennial habit of growth, leaf blades rolled in young shoots, a wider leaf blade, more florets per spikelet and taller mature plant height. Frakes (1973) also suggests that the ryegrasses can be grown on a wide range of soil types. However, if extended low

temperatures, drought and poor fertility are characteristic of the area to be seeded, ryegrasses may not be the most desirable species.

Lolium x Festuca Hybrids

Attempts are being made by researchers to incorporate genetic material from the highly nutritious Lolium spp. (ryegrass) to the hardy Festuca spp. (fescues). This is desirable since the fescues are capable of surviving in a wider range of temperatures than do the ryegrasses. Since the fescues make luxuriant fall pastures such an accomplishment will help to enhance their pasture potential.

To date, hybridization of Lolium x Festuca has been accomplished both in the United States and some areas in Europe. The hybrids tend to be infertile but Buckner et al (1961) found that in tall fescue x ryegrass hybridization, doubling the chromosome number with colchicine restores fertility. However, meiosis is not stable and chromosomes are eliminated during successive generations; as a result, progress in intergeneric hybridization by conventional grass breeding techniques is relatively slow. Hill and Carnahan (1962) found that Lolium x Festuca hybridization was generally more successful when Lolium was the maternal parent.

Buckner et al (1961) found that the F_1 Lolium x Festuca hybrids have foliage closely resembling that of the ryegrass parent. However, morphologically there was considerable variation among plants. They reported that F_1 hybrids of perennial ryegrass x tall fescue survived for 9 years and that of Italian ryegrass x tall fescue survived at least 6 years, thus indicating that the hardiness of the fescues are transmitted to the F_1 hybrids. A comparison of the palatability and

vigor of the F_1 hybrids with that of Ky31 showed that 37 of the 101 hybrids were superior in palatability and 53 were more vigorous. They also found that all 11 F_1 plants of Italian ryegrass x tall fescue were more vigorous and palatable than Ky31.

Genetics of Meadow Fescue, Tall Fescue, Ryegrass and Ryegrass x Fescue Hybrids

Meadow Fescue

Poehlman (1959) described varieties of meadow fescue with the diploid chromosome number 2n = 14, tetraploid chromosome number 2n = 28, hexaploid chromosome number 2n = 42 and decaploid chromosome number 2n = 70. Malik and Thomas (1967) suggest that in the formation of Festuca elatior complex, three species were probably involved, of which Festuca pratensis was one.

Tall Fescue

By contrast, Webster and Buckner (1971) state that tall fescue is a bivalent-forming auto-allohexaploid. Malik and Thomas (1967) also reported that tall fescue species had tetraploid (2n = 28) chromosome levels and decaploids (2n = 70) chromosome levels. Malik and Thomas (1967) who were sampling a collection of fescues from the French Alps, found one plant which contained the triploid chromosome number (2n = 21). They postulated that this was possibly a hybrid between Festuca pratensis (2n = 14) and Festuca arundinacea var. Glaucescens (2n = 28), because it was morphologically similar to artificial triploids.

Ryegrass and Ryegrass x Fescue Hybrids

Perennial ryegrass and Italian ryegrass are believed to be self-

incompatible but they can be crossed readily with each other. Both perennial and Italian ryegrasses have chromosome numbers of 2n = 14. Hybrids between diploid ryegrasses and auto-tetraploid ryegrasses with 2n = 28, also occur.

Chromosome numbers of ryegrass x fescue hybrids will vary depending on whether the parental material is meadow fescue x ryegrass or tall fescue by ryegrass.

Alkaloids of Fescue and Ryegrass

Definition of Alkaloids

Alkaloids, as defined by Pelletier (1970), are a group of compounds that have the following properties: 1) chemically basic; 2) nitrogen containing; 3) of plant origin; 4) significant pharmacological activity and 5) complex molecular structure. However, Robinson (1974) suggests that it is no longer justified to consider alkaloids as of plant origin only, since several animal products have all the necessary characteristics of alkaloids. Many authorities have reserved the name alkaloids for nitrogen compounds with complex molecular structure. Instead, simple amines are regarded as "protoalkaloids". Culvenor (1970) suggests that although alkaloids do not seem to play a role in plant nutrition or development, they may serve an ecological function.

Occurrence

The occurrence of "fescue foot" and poor animal performance of cattle grazing fescue pastures has motivated researchers to analyze the alkaloid content of this genus. Perloline was found to be the major alkaloid of fescue; two other alkaloids of lesser importance

are loline and perlolidine. Perloline was first discovered by workers studying abnormal pigments present in the basal shoots of ryegrass (Lolium perenne L.), which they suspected of causing facial eczema in sheep (Grimmett and Melville, 1943). White and Reifer (1945) also found perloline in reasonable concentrations in Festuca.

The alkaloids identified in fescues represent two widely divergent structures - perloline and loline (Figures 1 and 2, respectively). Normally, the alkaloids of a plant are members of the same chemical group and probably share a common biogenetic pathway. Since this is not the case in fescue, it is difficult to postulate a common precursor for perloline and loline biogenesis.

Biosynthesis of Perloline, Perlolidine and Loline

Perloline and perlolidine are members of the quinoline group of alkaloids and are the only two known natural products with a diaza-phenanthrene ring structure. Culvenor (1973) cites references which suggest a possible biosynthesis pathway for perloline. He postulates that perloline originates from tryptamine (assuming loss of C_2), a C_3 unit and an aromatic ring. The tryptamine and dihydroxyphenylalanine units combine to form a 2-arylquinoline derivative in which the 2-aryl group is subsequently transferred to the nitrogen (Figure 3).

Since the chemical structure of perlolidine is as shown in Figure 4, then it is justifiable to speculate that perlolidine follows basically the same biosynthetic pathway as perloline. One can conceive that loss of the veratrole moiety will result in the formation of perlolidine (Figure 5).

FIGURE 1. Structure of perloline.

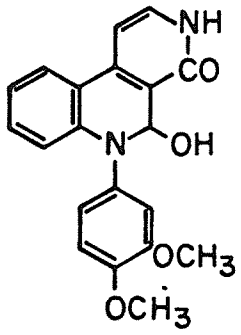

NH
CO
N
OH
OCH3
OCH3

FIGURE 2. Structure of loline.

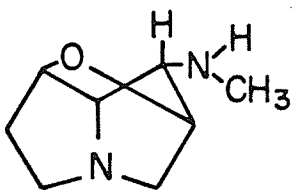

H
O
N
H
CH3
N

FIGURE 3. Suggested pathway for biosynthesis of perloline. Reproduced from Culvenor (1973).

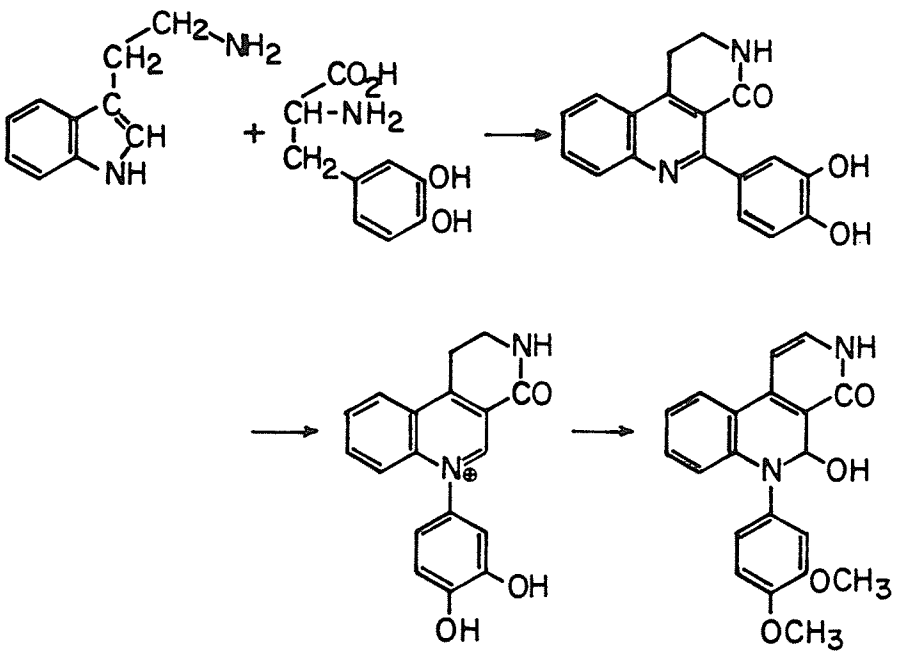

CH2
NH2
CH2
C
CH
NH
+
CO2H
CH-NH2
CH2
OH
OH
NH
CO
N
OH
OH
NH
CO
N⊕
OH
OH
NH
CO
N
OH
OCH3
OCH3

FIGURE 4. Structure of perlolidine.

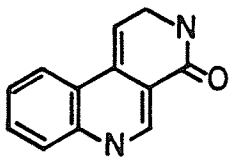
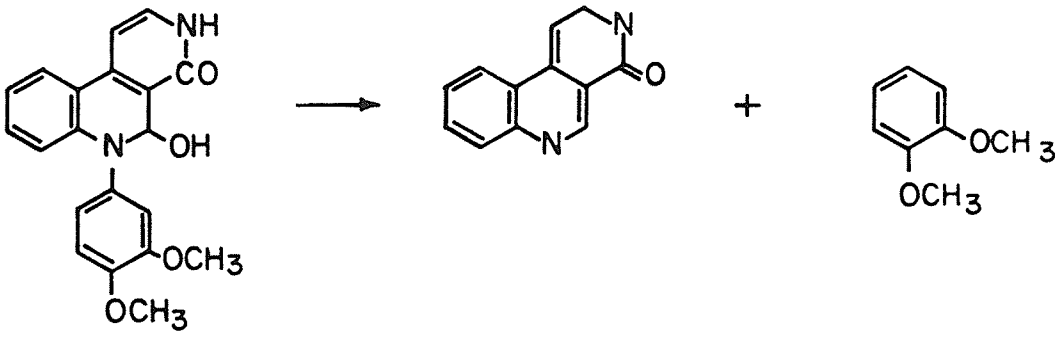

N
O
N

FIGURE 5. Possible formation of perlolidine.


NH
CO
N
OH
OCH3
OCH3
N
O
+
OCH3
OCH3

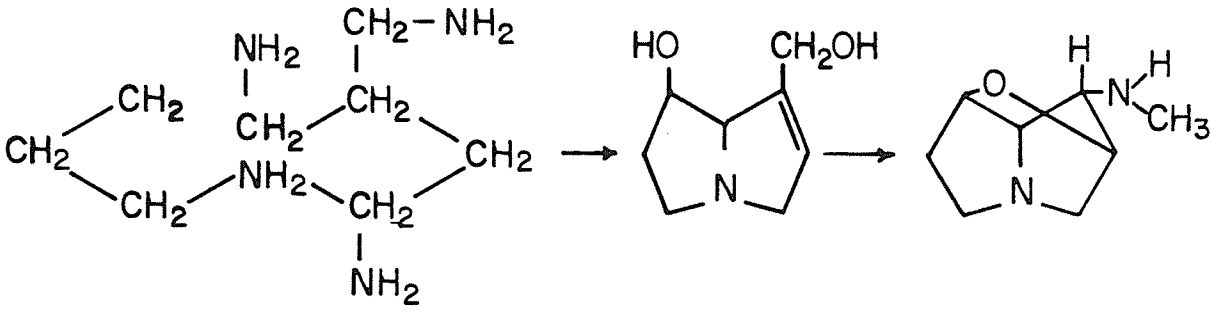
Biosynthesis of Loline

Loline is pyrrolizidine alkaloid which is composed of a pyrrolizidine ring. In addition it has a cyclic ether bridge and an attached secondary nitrogen (Figure 2). Bull et al (1968) suggest that contemporary studies indicate that the pyrrolizidine ring is built up from two 4-carbon units related to putrescine and ornithine as shown in Figure 6. Once the pyrrolizidine ring is formed, through further reactions the cyclic ether bridge is incorporated into the structure.

Distribution Within the Plant

Gentry et al (1969) reported the distribution of alkaloids in five strains of tall fescue, and the relationship of growth stage and growth conditions to alkaloid levels. They reported that the seeds of Ky31 and Kenwell varieties contain loline and two unknown alkaloids. Seeds of Alta, NK36 and Goar on the contrary possess no alkaloids. The alkaloid content of Ky31 and Alta seedlings increases over a period of 18 days from germination; most of the alkaloid is perloline. Perloline is the principal alkaloid in seedlings of all varieties. Ten-day-old seedlings of Ky31 and Kenwell contain six different alkaloids; while those of Alta, Goar and NK36 have only three. Total alkaloid levels of Ky31 are higher in the roots, shoots, stems and leaves than in the seeds and seedling heads. In all parts except seeds, perloline is the predominant alkaloid. The highest concentration is found in the roots of regrowth material and in the stems of plants at the dough stage. This presence of perloline in plant seedlings, but not in the seeds, indicates that the genetic template for perloline synthesis within the plant is present; but requires some form of catalyst either environmental or physiological,

FIGURE 6. Suggested biogenetic pathway of loline. Modified from Bull et al. (1968).


CH2
CH2
CH2
NH2
CH2
CH2
CH2
NH2
CH2
CH2
NH2
CH2
NH2
HO
CH2OH
N
O
H
N
H
CH3
N

in order to stimulate perloline production.

Factors Affecting Alkaloid Levels in Plants

The alkaloid levels of plants, though genetically determined, fluctuate considerably due to environmental influence. This has been substantiated by researchers who found that some non-toxic plants grown successfully in certain parts of the world, are very toxic when grown in other regions (Waller and Nowacki, 1978). This toxicity has been attributed to an increase in alkaloid content; other factors have also been implicated in the cause of noted increases in alkaloid levels. The effect of these factors on alkaloid concentrations are not the same for all plants. It has been reported by Waller and Nowacki (1978) that one factor may alter the biosynthesis or degradation of alkaloids of various origins; this same factor will increase the alkaloid production in one species and decrease it in another species. Some factors which influence alkaloid content are: i) variety of grass; ii) season of the year; iii) rate and type of fertilizer treatment; iv) water and general climatic conditions and v) fungal infection.

Variety of Grass

It has been observed that certain varieties will differ in their levels of alkaloid content. This is quite conceivable since alkaloid content is genetically controlled. Cornelius *et al* (1974) have found that the progeny of high-perloline parents are significantly higher in perloline than was progeny of low perloline parents. Buckner *et al* (1973), working with *Lolium* x *Festuca* hybrids, have also found that by increasing the ploidy level, there was a subsequent increase in perloline content.

Season of the Year

Gentry (1969) has studied seasonal variation in fescue alkaloids. He has observed that the total alkaloid content is low in winter, increases slowly through the spring and summer and reaches a maximum in July or August.

Rate and Type of Fertilizer Applied

The quantity and type of fertilizer applied have been found to influence alkaloid content in plants. Gentry et al (1968) cited by Culvenor (1973) noted a maximum perloline content of .37% for the tall fescue variety Kenwell. When this same variety is treated with N fertilizer at a rate of 112 Kg N/ha, the perloline content is .67%. Bush and Buckner (1973) found that tall fescue seedlings grown in greenhouses increased in perloline content as the level of nitrogen in the nutrient solution was increased. Gentry et al (1969) found that phosphorous and potassium combined greatly reduced perloline biosynthesis in tall fescue. However, Bennett (1963) found that the addition of phosphate alone or together with nitrate, had no significant effect on perloline levels. Waller and Nowacki (1978) report that in some species the addition of N will cause an initial increase in alkaloid content, after which further addition of N has no net effect on alkaloid levels. They further report that P and K have no effect on alkaloid content of some species.

Water and General Climatic Conditions

The effect of climatic conditions on alkaloid content as reported by Waller and Nowacki (1978) is shown in various fodder plants which are recognized in Europe as excellent forage grasses. For example,

when these were introduced into the semi-arid regions of Australia, Central Asia and the United States, they were found to be harmful in their new environment. Some of the best known plants subject to this change include fescues, loliums and reed canarygrass. Buckner et al (1973) report that the perloline content for meadow fescue in some areas of the United States can attain a level as high as 3,545 μg/gm dry matter. He attributes this to lack of adaptability of that variety to the new area where it is being grown. Further research also suggests that low moisture levels in some soils is the cause of increased alkaloid levels in plants.

Fungal Infection

The alkaloids and quantity of these produced may be directly influenced by fungal infection. For example, Tookey and Yates (1972) cite literature in which it is suggested that hay from tall fescue infected with Stemphylium spp. does not contain loline. Moreover, some related alkaloids may also be absent. Furthermore, leaf lesions caused by invasion by Rhizoctonia solani Kuhn. or Helminthosporium vagaas Dreen. have a reduced amount of perloline compared to healthy tissue. Comparison of varieties shows that genotypes which produce the most perloline when healthy, are most resistant to infection by R. solani; thus, their disease severity is less and is reflected in the greater amount of perloline appearing in the diseased plants.

Digestibility and Toxicity

Considerable work has been done by Bush and associates to determine the effect of perloline on rumen microflora. Bush et al (1970), using different cellulose sources, have studied perloline inhibition

of *in vitro* rumenal cellulose digestion. They have found that regardless of cellulose source, digestion is measurably inhibited by levels of perloline greater than 9.1×10^{-5} M. However, when perloline concentrations are below 7.4×10^{-4} M, greater inhibition is observed with purified cellulose as the substrate instead of tall fescue. Bush *et al* (1972) have further studied the inhibitory effects of perloline to rumen fermentation *in vitro*. Inhibition of *in vitro* cellulose digestion and total volatile fatty acid (VFA) production occurred at concentrations greater than 1.2×10^{-4} M perloline. The volatile fatty acids observed are: acetic, propionic, butyric, isovaleric and valeric. Compared to the control an actual stimulation of VFA production was observed up to 1.2×10^{-4} M perloline. It has also been observed that approximately 70% inhibition in cellulose digestion is required before VFA production is inhibited. Bush *et al* (1972) have suggested that this apparent stimulation in VFA production may be from utilization of soluble substrates in the fermentation media and not from cellulose. Studies by Bush *et al* (1972) also show that inhibition of *in vitro* cellulose and VFA production can be caused by an inhibition of specific biological processes or general acute toxicity to the rumen micro-organisms. They cultured four principal cellulolytic rumen bacteria under anaerobic conditions. Perloline was then added to the culture media. Results have indicated that as the concentrations of perloline in the culture media is increased, there is a marked decrease in bacterial population. Bush *et al* (1972) have, therefore, concluded that perloline inhibits cellulose digestion and VFA production by inhibiting growth of rumen cellulolytic bacteria.

Boling *et al* (1975) have studied the effect of added perloline on

nutrient digestibility and metabolism in lambs. They have found that in lambs fed with perloline-added diet, there is lower crude protein digestibility. This is attributed to a similar cause to that reported by Bush et al (1972) in which they postulate that perloline inhibits the growth of certain rumen cellulolytic bacteria. Thus, the apparent decrease in digestibility of crude protein could feasibly be because of partial inhibition of microbial protein synthesis from ammonia, derived from rumenal dietary proteolysis, and also to decrease fermentation of all fermentable dietary components.

Tookey and Yates (1972) have found in their research that loline has low oral toxicity and a single oral dose of 400 mg/Kg had no demonstrable effect on mice. However, if the 400 mg/Kg dose was given intravenously it caused convulsions and death. They also discovered that if a crude alkaloid mixture having loline as a major alkaloid were fed to a heifer at 16 g/day for 11 days, no toxicity was observed. They attributed this lack of toxicity to the saturated ring system in loline. Mattocks (1968) suggests that unlike the saturated ring system, the 1,2-unsaturated pyrrolizidine ring in toxic alkaloids is dehydrogenated in the liver to a pyrrole derivative, which is the true hepatoxin.

Bush et al (1976) have also studied the effect of perlolidine on digestibility. They have found that perlolidine has similar inhibitory effects on in vitro rumenal cellulose digestion and VFA production as did perloline.

Methods of Alkaloid Analysis

Several methods have been proposed for the extraction and quantification of alkaloids. However, because of the wide diversity in the

different classes of alkaloids, a method that has been used successfully for the extraction of one type of alkaloids will not necessarily work in extracting another. Thin layer chromatography and paper chromatographic methods are available for the separation and quantification of fescue alkaloids. Although they are considered slow, they are the only methods that have been used with overall satisfactory reproducibility *(Yates, 1978). Gas chromatography has also been used for separation of fescue alkaloids. However, because of perloline denaturation at such temperatures, it is difficult to achieve reproducible quantification for perloline. For the loline with the exception of the amine forms which tend to tail on OV columns, separation is acceptable *(Yates, 1978). Another disadvantage of gas chromatography is the relatively long and critical clean-up procedure which also makes this method extremely slow.

Choice of Absorbent Layer and Solvent System for TLC

In TLC, compounds which are being separated behave differently depending on the nature of the adsorbent layer. As a result, the resolution obtained varies. The nature of the solvent system also influences the resolution that will be obtained. Thus, it is usually necessary to try various adsorbent layers and solvent systems to determine which is best suited. However, investigations by researchers in the United States showed that TLC using Silica Gel G as the adsorbent layer, coupled with a solvent system of butanol-acetic acid saturated with water (4:1:5) worked best for the separation of fescue alkaloids.

*Personal communication.

Bush and Jeffreys (1975) found that employing the butanol system in TLC was the best way to separate crude extracts of tall fescue and ryegrass. They found that with this system pigments which normally interfered with other chromatographic systems, moved near the solvent front and the alkaloids could be readily seen.

Chromatography plates used in this experiment were commercial Analtech and Whatman plates obtained from Terochem Laboratory Supplies, Winnipeg, and as a result, there was no need for activation of the plates. The zone recovery pipet was obtained from Fischer Scientific. All pure alkaloids used were supplied by Drs. L.P. Bush and S.G. Yates of the United States.

MATERIALS AND METHODS

The material examined in this research included established cultivars of Meadow fescue grown in Canada plus introduced cultivars primarily of European origin (Table 1). Two Lolium x Festuca entries were also studied.

The site for this experiment was located on Campus at the University of Manitoba. The location used is known to have a heavy winter snow cover and because of the gradient, it is usually very wet until early summer. The soil type is clay and prior to planting, nitrogen was added at the rate of 30 Kg/ha.

Seeds of cultivars to be screened were pre-germinated in petri-dishes in the laboratory before being planted singly in Jiffy pots placed in flats. The plants were kept in the greenhouse for 2 months, then transferred to a cold-frame for a 1 wk acclimatization period before being transplanted to the field. They were planted in a completely randomized design with .914 m spaces within and between rows. This area was then irrigated immediately after planting to aid establishment of the plants.

Plants were sampled every 2 wks, taking a combination of leaf and stem material. The same area of the plant was always used in the sampling. In order to maintain enough material to accommodate such a sampling interval, the quantity of material taken at each interval was kept to a minimum. Sampled plant material was oven-dried at 50°C and

TABLE 1. List of 34 meadow fescue and two *Lolium* x *Festuca* hybrids included in the alkaloid study.

Cultivar	Country of origin	Ploidy level
Festina	Netherlands	Diploid
Vagones		"
Merbeem (R.V.P.)	Belgium	"
Mana	Denmark	"
Belimo	Netherlands	"
Garanta	Netherlands	"
Paavo	Finland	"
Afu	Netherlands	"
Cykado		"
Wendelmoed	Netherlands	"
Barbarossa	Netherlands	"
Dufa	Netherlands	"
Bundy	Netherlands	"
Janetzki	Federal Republic of Germany, Origin Netherlands	"
Senu	Sweden, and now Denmark	"
Barkas	Netherlands	"
Havingull	Iceland	"
Largo	Netherlands	"
Perdita	Great Britain	"
Cosmos II	Federal Republic of Germany	"
Trader	Canada	"
Rossa (Hay type)	Netherlands	"
Bergamo	Netherlands	"
Bottnia II	Sweden	"
Loken	Norway	"
Sequana	France	"
Aberystwyth S53	Great Britain	"
Tammisto	Finland	"
Ensign	Canada	"
Landis MF-001		"
MSU		"
Svalof (Sena)	Sweden	"
Mimer	Sweden	"
Prior (PR x MF)		Tetraploid
Elmet (IR x MF)		"
Angsvingel		Diploid

ground to a texture which was able to pass through a 2 mm sieve in preparation for alkaloid analysis.

Chemical Analysis

The objective of this experiment was to find a suitable method that would not only permit the handling of a large number of samples but would also do so with acceptable efficiency. Therefore, the methods attempted for the extraction and quantification of fescue alkaloids included those used by Gentry et al (1969), Woods and Clark (1971) and Hultin and Torssell (1965).

Woods and Clark Method

This method, perfected by Woods and Clark (1971), to extract and to quantify reed canarygrass alkaloids is as follows: approximately 5-10 gm of frozen grass were chopped into pieces 1-3 cm long and steeped overnight in 100 ml of a mixture of chloroform, methanol and concentrated ammonium hydroxide (26:33:1). It is believed that freezing of the grass will rupture the cells thereby enhancing extraction efficiency. Duplicate samples of 10 ml were then removed and purified in large test tubes by the following procedure: 10 ml of 2N H_2SO_4 are added; the contents stirred and the pigmented chloroform layer discarded by using an aspirator system. Ten more ml of chloroform are added and after settling has occurred the chloroform layer is removed. Ten millilitres of water are added; the contents stirred and the aqueous layer is again discarded. This leaves 10 ml of chloroform containing the extracted bases. The remainder of this method is designed for the analysis of hordenine and gramine and is, therefore, not applicable to the present study.

Though chromatography was not included in the original method used by Clark and Woods, it was included as part of this research. Twenty microlitres of the chloroform containing bases were spotted on TLC plates using Silica Gel G. as the adsorbent layer and developed in butanol, acetic acid and water (4:1:5). Bases were identified using a pure standards. Because of the availability of pure alkaloid standards Rf values were not used to identify alkaloids in this research. However, the TLC Rf values as reported by Bush and Jeffreys (1975) for perloline and perlolidine were .23 and .70 respectively, when Silica Gel G. was used as the adsorbent layer and the butanol system was used; no Rf values were found for loline.

Gentry et al Method

The method used by Gentry et al (1969) is as follows: 1 gm of oven dried plant material is mixed with .25 gm of sodium bicarbonate and 1 gm of neutral acid-washed sand in a mortar; 2 ml of distilled water are added and the mixture is then ground thoroughly for 2 min. The alkaloids are extracted with 10 ml of chloroform to which 5% by volume of ethanol had been added. The mixture was ground for 30 seconds and covered immediately to prevent evaporation. After 15 mins the solution was separated from the residue by filtration. Twenty microlitres of the filtrate were spotted on a thin-layer chromatography plate using Silica Gel G as the adsorbent layer, and developed with butanol-acetic acid saturated with water (4:1:5). Potassium iodoplatinate color reagent was used to locate the various alkaloids. These were then scraped from the plates and eluted from the adsorbent with 0.05 N HCl. The suspension was centrifuged by using a bench model centrifuge and

then filtered. The alkaloid concentration was then determined spectrophotometrically using a Bausch and Lomb Spectronic 20 spectrophotometer.

Modified Method of Hultin and Torssell (1965)

Sampled plant material was dried in an oven at 50°C for at least 24 hr; the material was then ground to pass through a 2 mm sieve. Four grams of this oven dried plant material were steeped overnight in 40 ml of methanol. The mixture was filtered and the residue is then rinsed with 20 ml of methanol, combining both filtrates in an evaporatory flask; the combined filtrate was then evaporated to dryness using a Buchi Rotavapor-R. The residue was resuspended in 2 ml methanol; the solution was filtered to remove any clumps of material and 20 µl of this filtrate were spotted on a thin layer chromatography plate which uses Silica Gel G at 250 micron thickness as the adsorbent layer. This chromatogram was developed in butanol-acetic acid saturated with water (4:1:5). All chromatograms were allowed to run 15 cm approximately 6 hr. The plates were then removed and air dried. Prior to spraying, perloline is discernible as a yellow spot; when viewed under U.V. light, it has a greenish fluorescence. The plate was then sprayed with potassium iodoplatinate to develop the other spots. Each individual alkaloid sought was identified by using its pure form as an indicator spot.

Perloline was scraped off the plate by using a TLC zone recovery pipet to which an additional piece of filter paper was fitted over the sintered filter disc. The filter paper containing the removed spot was then placed in a centrifuge tube to which was added 10 ml of 0.05 N HCl as an eluant. The tube was then shaken to suspend the Silica Gel particles. Following this, the suspension was centrifuged to sediment

the particles. The eluate was then filtered and perloline concentration in the filtrate was determined spectrophotometrically with a Bausch and Lomb Spectronic 20 at 395 nm. The concentration was calculated using a standard curve.

Preparation of Potassium Iodoplatinate

Three millilitres of 10% platinum chloride solution was mixed with 97 ml of water to which was added 100 ml of a 6% aqueous solution of potassium iodide.

Statistical Analysis

The t-test for significant differences in means was used to determine whether or not the means obtained were significantly different from 1000 µg/gm dry matter. Following which Tukey's test was employed to show significant differences in all means less than 1000 µg/gm dry matter. All calculations were done using the means of the five replicates per cultivar.

RESULTS AND DISCUSSION

Discussion of Methods

Of the three methods attempted for use in this research, the modified method of Hultin and Torssell (1965) was considered to fulfill best the initial aim of the project. As a result, this method was used for all qualitative and quantitative analysis carried out.

Woods' and Clark's (1971) method provided for the handling of the largest number of samples. Although this method was efficient for the analysis of reed canarygrass alkaloids, it was inappropriate for fescue alkaloids. Firstly, the quantity of methanol used in the extraction procedure allows for the loss of minor alkaloids in the discard. This was verified when loline and perlolidine samples were added to crude grass extract but were unnoticed in the chromatographic procedure added to the method. Secondly, Dr. S.G. Yates, Peoria, Illinois (1978) has suggested that perloline is unstable in unwashed dry chloroform solutions.

It was necessary to add a chromatographic step to this method for the following reasons: perloline is a "yellow" alkaloid which discolours the solution yellow with a greenish fluorescence. Thus, it is possible to read perloline concentration spectrophotometrically after the crude extraction is completed. However, because it is a crude extract, there are pigments besides alkaloids which may be present. Some of these pigments such as flavonoids absorb at or near to the

absorption maxima of perloline in chloroform. Thus, any attempt to read the perloline content directly in the crude extract will result in a reading higher than the true value, due to spectral overlap. Therefore, there is a need to separate these compounds before any attempt could be made to quantify them. Furthermore, because the poor solubility of loline in chloroform, it is lost in the discard at this step. Likewise, other minor alkaloids are also lost at various steps. Therefore, chromatography serves no purpose except for separating pigments from alkaloids. Thus, it does not enhance the suitability of the Woods and Clark method for the extraction of fescue alkaloids.

The method devised by Gentry et al (1969) can be employed successfully in the study of fescue alkaloids and except for the extraction procedure, it is similar to the modified one of Hultin and Torssell (1965). However, the method was incapable of handling a sufficient number of samples per day, and therefore it could not be used in a screening program.

Hultin and Torssell (1965) enhanced the extraction process by grinding the material mechanically and by allowing the extraction to take place overnight. Their technique thus permitted the handling of a larger volume of samples, allowed for consistency in extraction and for the reduction in determinate errors.

Running the chromatograms for approximately 6 hr allowed the removal of most of the tailing substance close to the desired alkaloids. In view of the fact that the alkaloids sought were identified only by using a pure alkaloid standard as the indicator spot, and not by R_f values, then the risk of the solvent running off the absorbent layer was not critical since the alkaloids sought had low R_f values.

General Observations

It was observed that with the exception of the ryegrass x fescue hybrids, all the remaining cultivars were affected by rust; the severity of the infection varied with the species. No attempt was made to correlate alkaloid content to the degree of the infection. It was felt that the susceptibility of each species to rust can be better correlated to alkaloid content, if it were done under controlled conditions.

Because meadow fescue has a slow recovery rate relative to other forage species, sampled plant material with the exception of the ryegrass x fescue hybrids and the two Canadian synthetic varieties were slow in recovering. Overall plant growth declined and in some areas some of the tillers of some cultivars died. The ryegrass x fescue hybrids and the Canadian cultivars had fairly lush growth at all times.

Plants of the cultivars observed were qualitatively screened for alkaloid content in the summer of 1978. One sampling date was arbitrarily chosen in mid-July because it was at this time that the plants of the newly established nursery had attained sufficient growth for sampling. Qualitative analysis was done using the modified method of Hultin and Torssell (1965) and evaluation based on grass samples provided by L.P. Bush of Kentucky. These grass samples were determined by him to be low perloline, high perloline, low loline, high loline. Results showed a perloline content that ranged from "zero" - very high. Of the 36 cultivars observed, 19 showed "zero" - low perloline. In all cases loline appeared intermediate and perlolidine was not observed. Testing of the method to observe its ability to detect perlolidine showed that when a pure perlolidine standard was added to the sample at the stage of extraction, perlolidine was detectable using potassium

iodoplantinate spray reagent. Based on these qualitative results, plants were then quantitatively screened in the summer of 1979 for perloline only.

Because there was no apparent variation in loline content in plants of the same cultivar or different cultivars, the quantification of loline was considered to be unnecessary, as the objective of this research is to discover plants with a variation in alkaloid content so that they may be used in a breeding program to upgrade the present cultivars grown in Canada. Since there is no apparent variation in loline content, then there is no basis for using it as a tool for selection. No apparent relationship between loline content and perloline concentration was observed. This is conceivable since loline and perloline belong to a different group of alkaloids and share no apparent common biogeneric pathway. Because the method used is capable of detecting perlolidine, should it be present in future testing, then quantification could be done at that time.

The quantitative results obtained in the summer of 1979 were unexpected. There were no significant differences in the perloline content in individual plants of the same cultivar (see Appendix Tables 1 to 6).

The observed lack of variability of perloline levels in plants within a cultivar, is quite unusual and unexpected. Because these plants are synthetics, there should be considerable variation in the genetic pool, therefore variation in the alkaloid content. The obtained lack of variation in the alkaloid content of the individual plants may be accounted for on the basis of not having used a larger number of replicates for each individual cultivar. In this research, only five

replicates per cultivar were used in order to permit the screening of more cultivars.

Because there was an observed lack of variation in perloline content of the five replicates per cultivar used for all cultivars, the mean of the five replicates was used in reporting the perloline content for each cultivar.

If there was significant variation in the perloline content of individual plants within a cultivar, then it would not have been justifiable in using the means of the replicates in reporting the perloline concentration for the sampling date for a given cultivar. The reason being that, assuming two plants each had a perloline concentration of 150 μg/gm dry matter and the remaining three of the same cultivar each had a concentration of 400 μg/gm, the mean of the five replicates is 300 μg/gm. However, if in another cultivar each of the five plants had a perloline concentration of 300 μg/gm, the mean is still 300 μg/gm but the variation of the perloline concentration within cultivars are not the same. Had this situation occurred, then the selection of plants would have had to be based on an individual plant basis rather than cultivar. Using the individual plant in the selection process, this material could be cloned for enlargement of a nursery in order to study the general agronomic potential of the individual plant of that cultivar.

The concentration of perloline in the meadow fescue cultivars and meadow fescue x ryegrass hybrids sampled, showed significant variation between the period June 16 to August 7. The average perloline concentration ranged from 0 - 6051.2 μg/gm dry matter (see Figures 7 to 12). Maximum perloline concentration occurred in most cultivars tested on July 4; the perloline concentration in the remaining cultivars peaked

on July 18 as shown in Figures 7 to 12.

The general pattern obtained for the perloline content of the cultivars observed showed a relatively low initial perloline content on the first sampling. This was followed by a distinct increase in the perloline concentration in the third sample. For some varieties, this marked the maximum perloline level attained; in other cultivars, the rise in perloline content continued until the time of the fourth sampling. In the subsequent sampling, the perloline concentration in all cultivars fell off remarkably (Figures 7 to 12).

Seventeen of the 36 cultivars observed contained perloline levels <1000 µg/gm dry matter (0.1%). This level (0.1%) was suggested by Webb (1972) as being the maximum level at which there is seemingly no influence on animal performance. A comparison of the cultivars quantitatively determined to contain perloline levels <0.1% to those qualitatively determined to be low in perloline content, showed that the qualitative method was a reliable predictor in 85% of the samples tested. The two cultivars AFU and Festina, which were qualitatively determined to be low but quantitatively determined to be high, contained maximum perloline levels of 1006 µg/gm and 1350 µg/gm dry matter respectively.

Of the 36 cultivars used, Ensign and Trader were the only two Canadian synthetic varieties. Ensign, licensed in 1944 and Trader licensed in 1964 were developed from various European varieties. Therefore, one can conceive that those two varieties may have some genetic relationship to the others used.

Trader was observed to have a maximum perloline level of approximately 0.04% which is significantly lower than the maximum safe level

of 0.1%. Ensign contained a maximum level four times greater than the highest acceptable level. This variation in alkaloid content between the two Canadian synthetic varieties suggest the existence of great differences in the genetic pool of the two varieties, indicating that they were developed from very different cultivars.

A comparison of the cultivars which were quantitatively found to be <0.1% showed that within these varieties there are significant variations in the means of some cultivars (Table 2). Such significant findings are important only if the agronomic qualities of these low perloline lines are relatively equal. If these cultivars possessed similar agronomic qualities, then the level of perloline relative to each other, would be useful in the choice of lines for a breeding program.

No attempt was made to relate perloline levels to stage of growth of the plants. It has already been documented by previous researchers that the level of perloline increases with maturity; then there is a decline. In this experiment, perloline analysis was made on a regrowth basis; this was more applicable since it has been reported that perloline levels are higher in regrowth material. It was felt that the objectives of this research would be better fulfilled if the plants were subjected to conditions conducive to high perloline concentrations. Moreover, in view of the fact that this grass is primarily used as pasture, animals grazing this material would be consuming regrowth material at all times.

FIGURE 7. Plot of mean perloline concentration µg/g dry matter vs sample dates for five meadow fescue cultivars and one meadow fescue x perennial ryegrass hybrid.

*Perennial ryegrass x meadow fescue hybrid.

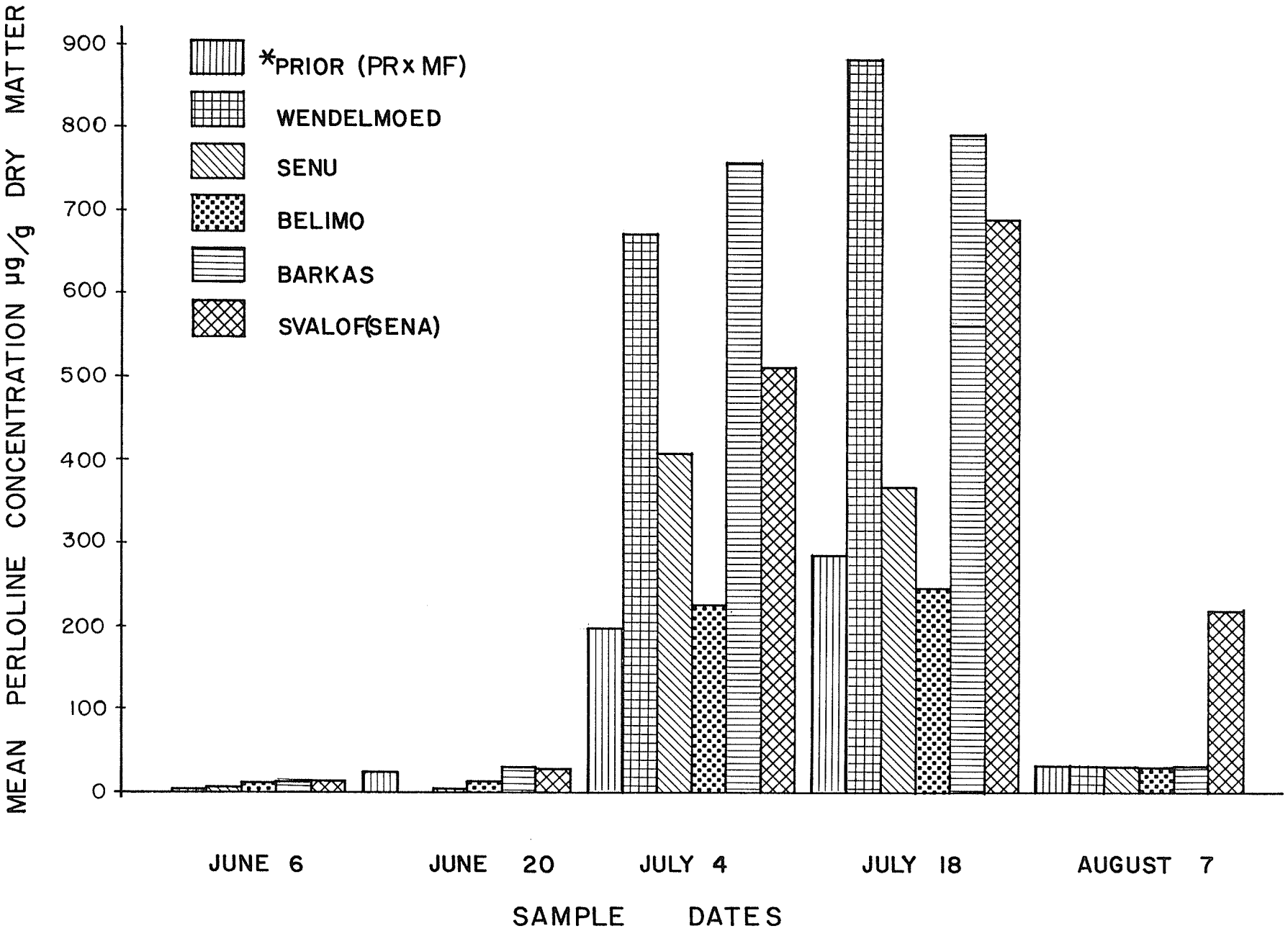

MEAN PERLOLINE CONCENTRATION µg/g DRY MATTER
900
800
700
600
500
400
300
200
100
0
*PRIOR (PR x MF)
WENDELMOED
SENU
BELIMO
BARKAS
SVALOF(SENA)
JUNE 6
JUNE 20
JULY 4
JULY 18
AUGUST 7
SAMPLE DATES

FIGURE 8. Plot of mean perloline concentration µg/g dry matter vs sample dates for six meadow fescue cultivars.

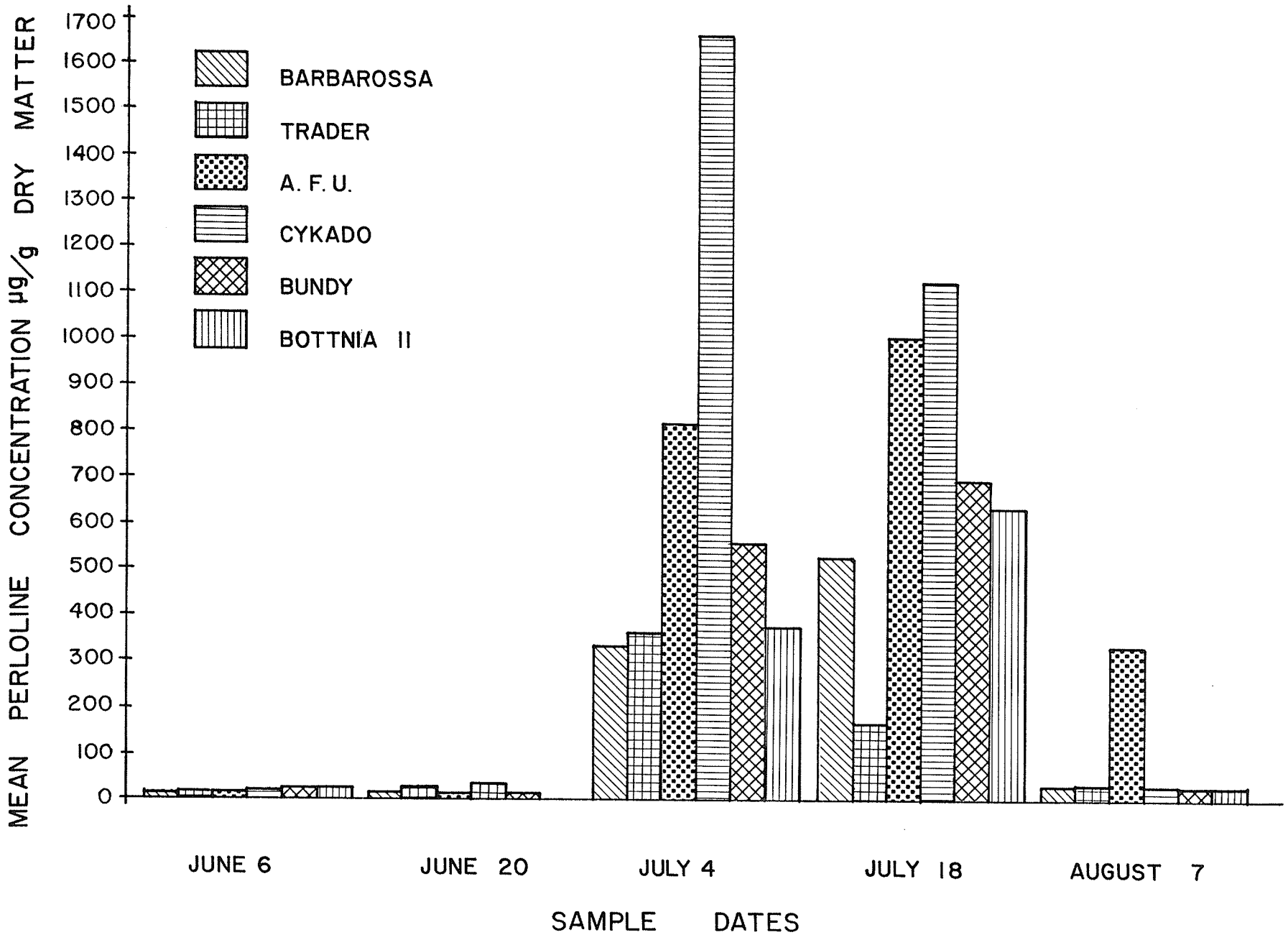

MEAN PERLOLINE CONCENTRATION µg/g DRY MATTER
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
BARBAROSSA
TRADER
A. F. U.
CYKADO
BUNDY
BOTTNIA II
JUNE 6
JUNE 20
JULY 4
JULY 18
AUGUST 7
SAMPLE DATES

FIGURE 9. Plot of mean perloline concentration µg/g dry matter vs sample dates for five meadow fescue cultivars and one meadow fescue x perennial ryegrass hybrid.

*Italian ryegrass x meadow fescue hybrid.

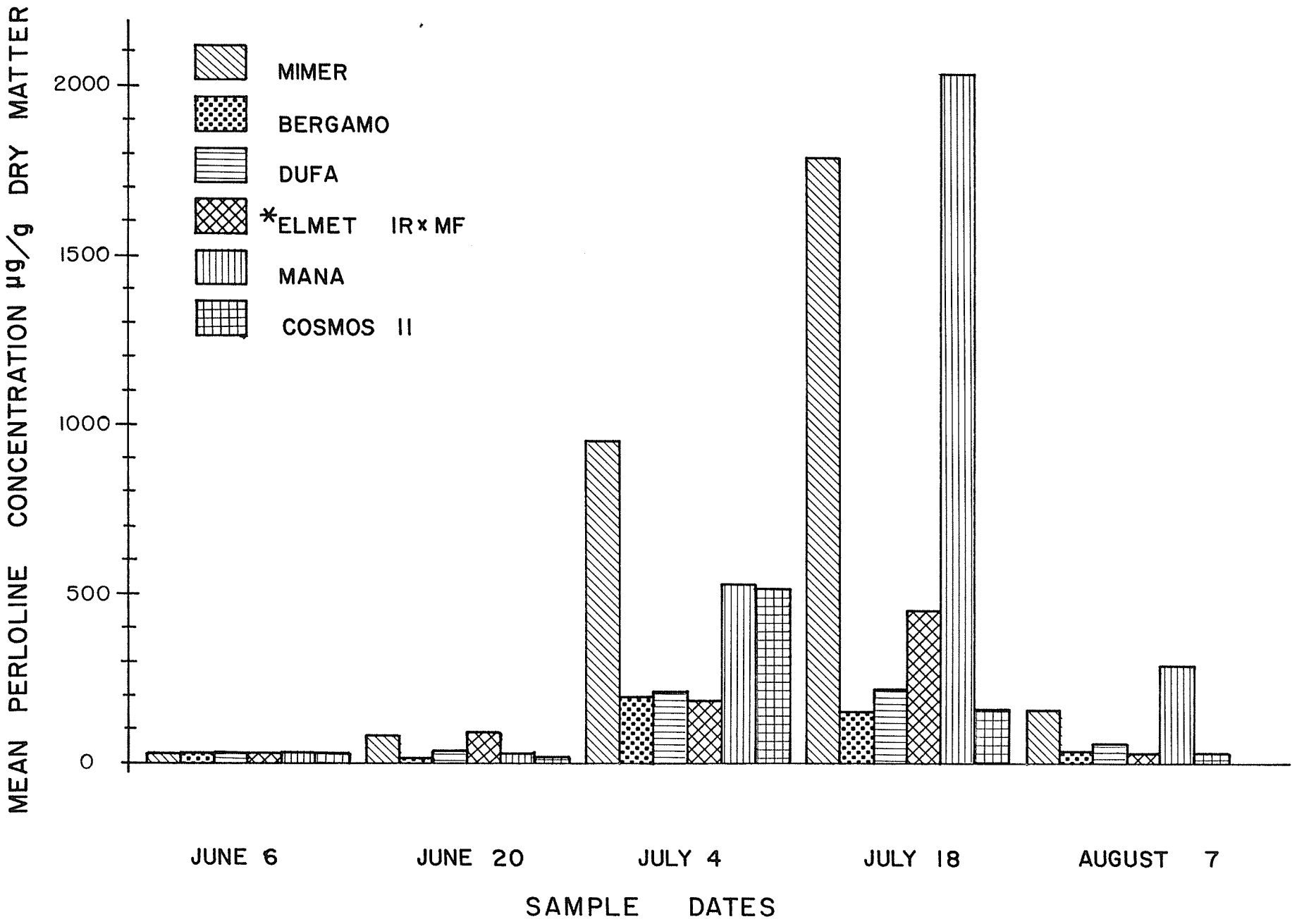

MEAN PERLOLINE CONCENTRATION µg/g DRY MATTER
2000
1500
1000
500
0
MIMER
BERGAMO
DUFA
*ELMET IR x MF
MANA
COSMOS II
JUNE 6
JUNE 20
JULY 4
JULY 18
AUGUST 7
SAMPLE DATES

FIGURE 10. Plot of mean perloline concentration µg/g dry matter vs sample dates for six meadow fescue cultivars.

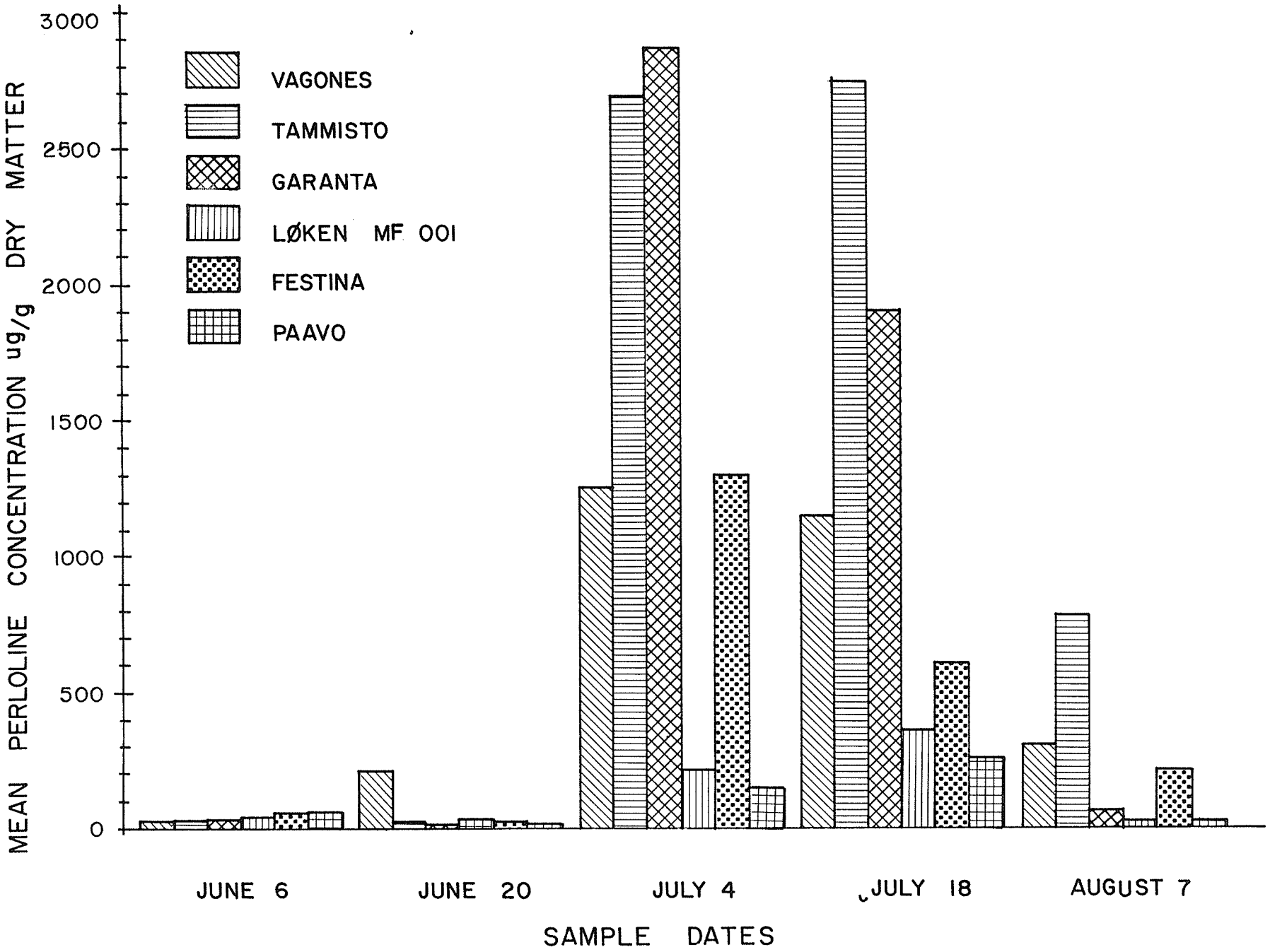

MEAN PERLOLINE CONCENTRATION ug/g DRY MATTER
3000
2500
2000
1500
1000
500
0
VAGONES
TAMMISTO
GARANTA
LØKEN MF 001
FESTINA
PAAVO
JUNE 6
JUNE 20
JULY 4
JULY 18
AUGUST 7
SAMPLE DATES

FIGURE 11. Plot of mean perloline concentration µg/g dry matter vs sample dates for six meadow fescue cultivars.

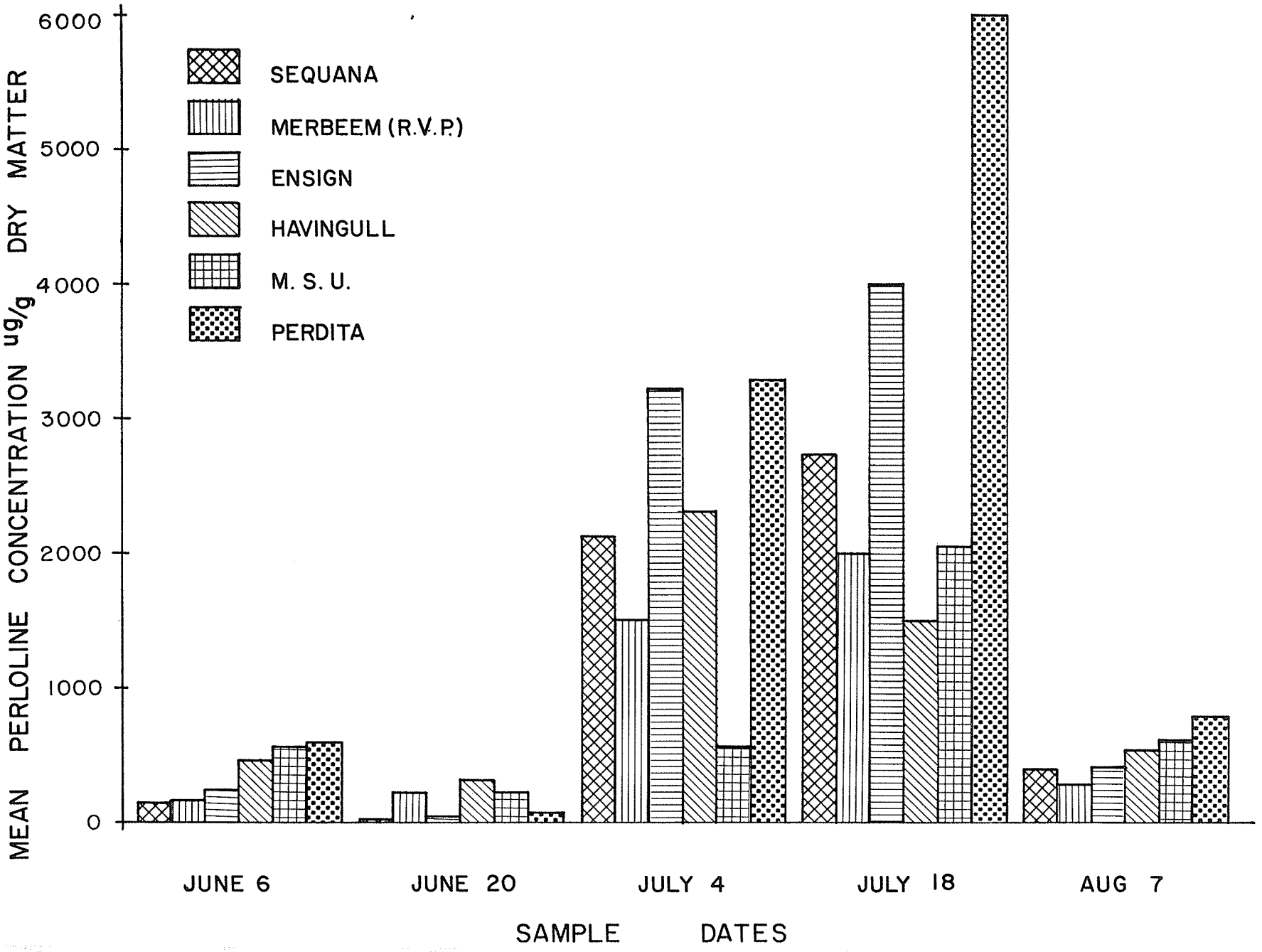

MEAN PERLOLINE CONCENTRATION ug/g DRY MATTER
6000
5000
4000
3000
2000
1000
0
SEQUANA
MERBEEM (R.V.P.)
ENSIGN
HAVINGULL
M. S. U.
PERDITA
JUNE 6
JUNE 20
JULY 4
JULY 18
AUG 7
SAMPLE DATES

FIGURE 12. Plot of mean perloline concentration µg/g dry matter vs sample dates for six meadow fescue cultivars.

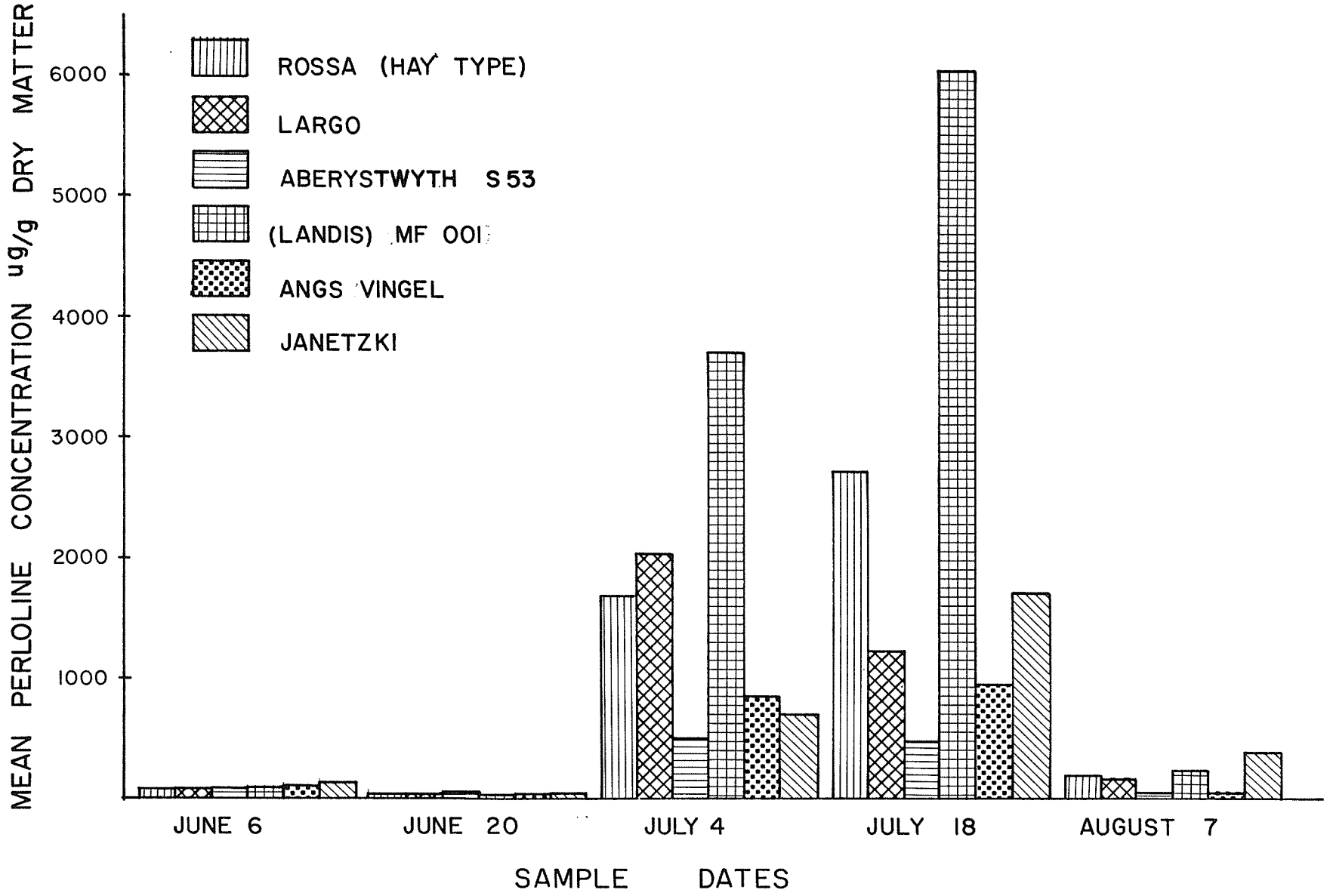

MEAN PERLOLINE CONCENTRATION ug/g DRY MATTER
6000
5000
4000
3000
2000
1000
ROSSA (HAY TYPE)
LARGO
ABERYSTWYTH S 53
(LANDIS) MF 001
ANGS VINGEL
JANETZKI
JUNE 6
JUNE 20
JULY 4
JULY 18
AUGUST 7
SAMPLE DATES

TABLE 2. Test for significant differences of cultivars containing a perloline level of 1000 µg/g dry matter.

Cultivar	Highest perloline level attained	Tukeys test for significant difference
Bergamo	196.6	a
Dufa	215.8	b
Belimo	242.2	c
Paavo	266.8	d
Prior (PR x MF)	276.8	e
Loken	359.8	f
Trader	359.8	f
Senu	406.2	g
Elmet (IR x MF)	452.2	h
Aberystwyth S53	499.5	i
Cosmos II	509.8	j
Barbarossa	526.4	k
Bottnia II	634.6	l
Bundy	694.2	m
Barkas	786.8	n
Wendelmoed	877.4	o
Angsvingel	940.0	p

PR x MF = Perennial Ryegrass x Meadow Fescue
IR x MF = Italian Ryegrass x Meadow Fescue

*Cultivars with corresponding letters are not significantly different at the .05 level of significance.

SUMMARY AND CONCLUSIONS

Although sampling every 2 wks is not typical of a pasture situation and may appear as an aggravated analysis, it was done primarily to get a close estimate of the possible peak date of perloline in the samples examined. It was believed that even though previous studies by researchers suggest that perloline concentration is highest in regrowth material, this increased perloline concentration would not be sufficient to cause the discarding of plants that are borderline in view of the fact that any decision made in the selection of the plants would have to be based on their overall agronomic potential and not primarily on perloline content.

The need to collect samples around the approximate peak time of perloline concentration in individual cultivars was to eliminate unnecessary analysis. If the peak time were known, then one sample could be taken at that moment and analyzed. Even if the peak dates reported in this research were inconclusive, samples for perloline analysis around the middle of July would give a relatively good approximation of the period of the greatest concentration of perloline. Based on the comparative results of the qualitative and quantitative methods of estimation, it thus appears feasible that the use of the qualitative method of analysis for further research is acceptable. The efficiency of this method of analysis could be enhanced if at the time of chromatographing the samples, a separate 1000 µg (0.1%) of pure perloline

sample is also applied and run on the same chromatograph. Any spot that has an intensity greater than the 0.1% spot should be rejected.

Having undertaken this research on plants grown at one location, there appears a need to repeat the project at other locations before any conclusive appraisal can be reached, because alkaloids like most agronomic qualities of plants are environmentally influenced. As a result, plants which were found to be acceptable at one location may be found to be unacceptable when cultivated in another areas. This was exemplified in the ryegrass x fescue hybrids used in this study. When they were grown on the plot on campus, their winter hardiness was excellent. However, when this cultivar was grown off campus at the Glenlea Research Station, their winter hardiness was approximately "zero".

In conclusion, it must be stressed that although the effect of alkaloids on the performance of animals is undoubtedly of great concern, these alkaloids form only a part of the whole in a grass breeding program. It, therefore, becomes necessary that in selecting plants to be used for this purpose, they be selected for their overall forage potential. It is, therefore, necessary to evaluate cultivars for their forage production, seed production, hardiness, disease resistance, chemical composition and alkaloid content at several locations for the best results. Moreover, because it has been suggested that low perloline plants may be more susceptible to disease, it would be necessary to research this further. For example, it was observed in this research that ryegrass x fescue hybrids were free of disease although they contained low perloline alkaloids.

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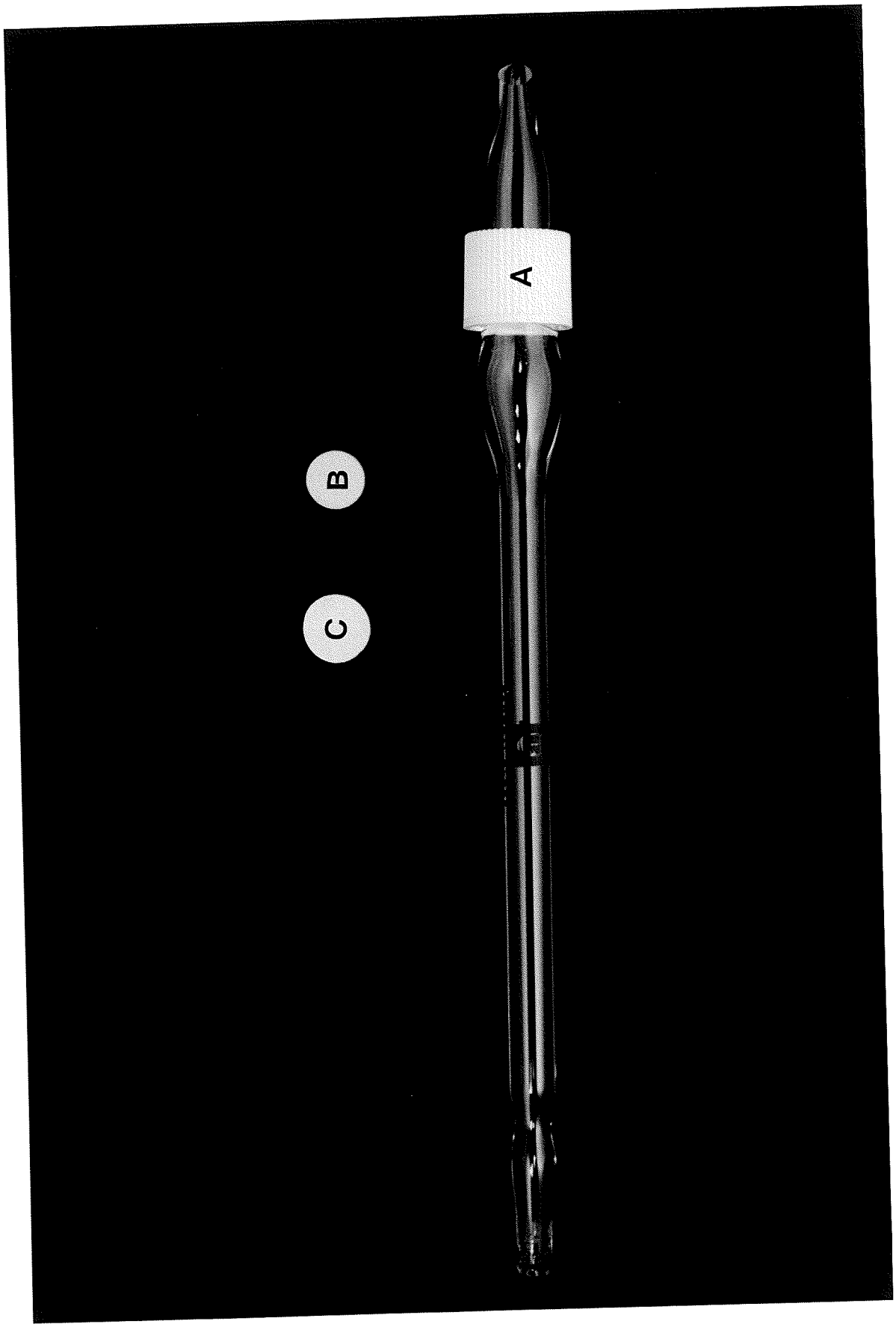
APPENDIX

APPENDIX FIGURE 1. Photograph of TLC zone recovery pipet.

A = Zone recovery pipet.
B = Sintered glass filter.
C = Filter paper.


A
B
C

APPENDIX TABLE 1. Perloline concentration of plants sampled June 6, 1979.

Varieties	Replicates Perloline Concentration µg/gm Dry Matter					$\bar{X}$ Reps	Variance	Std. Dev.
	1	2	3	4	5			
Prior (PR x MF)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wendelmoed	0.0	3.0	1.0	0.0	2.0	1.2	1.7	1.3
Senu	0.0	3.0	1.0	5.0	1.0	2.0	4.0	2.0
Belimo	10.0	11.0	7.0	10.0	8.5	9.3	2.4	1.6
Barkas	10.0	12.0	10.0	7.0	9.0	9.6	3.3	1.8
Svalof (Sena)	10.0	10.0	11.0	9.0	13.0	10.6	2.3	1.5
Barbarossa	10.0	13.0	10.0	9.5	11.0	10.7	2.0	1.4
Trader	10.0	9.0	12.0	10.0	13.0	10.8	2.7	1.6
AFU	10.0	13.0	11.0	8.0	15.0	11.4	7.3	2.7
Cykado	14.6	15.3	16.0	12.0	13.0	14.2	2.7	1.6
Bundy	20.6	23.0	19.0	17.0	20.2	20.0	4.8	2.2
Bottnia II	20.6	21.0	19.0	18.5	23.0	20.4	3.2	1.8
Mimer	26.8	24.2	22.0	23.0	29.0	25.0	8.2	2.8
Bergamo	26.8	24.5	25.0	22.0	29.0	25.4	6.8	1.6
Dufa	26.8	27.2	25.0	23.0	28.0	26.0	4.0	2.0
Elmet (IR x MF)	26.8	25.3	25.0	23.0	30.0	26.0	6.8	2.6
Mana	26.8	27.0	26.8	24.2	33.0	27.6	10.6	3.2
Cosmos II	26.8	29.0	27.0	26.8	30.0	27.9	2.2	1.4
Vagones	32.8	33.0	32.8	30.0	29.0	31.5	3.5	1.8
Tammisto	39.0	40.0	42.0	38.0	35.0	38.8	6.7	2.6
Garanta	39.0	42.0	37.0	36.0	43.0	39.4	9.3	3.0
Loken	45.0	47.0	45.0	41.0	43.0	44.2	5.2	2.2
Festina	57.0	57.0	53.0	55.0	54.0	55.2	3.2	1.8
Paavo	57.0	56.0	57.0	53.0	59.0	56.4	4.8	2.2
Rossa (Hay type)	69.5	73.0	65.0	70.0	69.0	69.3	8.2	2.8
Largo	69.5	72.0	70.0	69.5	71.0	70.4	1.2	1.0
Aberystwyth S53	75.5	76.5	71.0	72.0	74.0	73.8	5.3	2.3
MF-001 (Landis)	81.5	83.0	79.0	87.0	82.0	82.5	8.5	2.9
Angsvingel	87.5	92.0	88.0	79.0	90.0	87.3	24.7	5.0
Janetzki	130.0	132.0	130.0	128.0	129.0	129.8	2.2	1.5
Sequana	142.0	143.0	139.0	141.0	145.0	142.0	5.0	2.2
Merbeem (R.V.P.)	154.0	155.0	150.0	149.0	143.0	150.2	22.7	4.8
Ensign	222.0	224.0	220.0	213.0	217.0	219.2	18.7	4.3
Havingull	453.0	454.0	448.0	450.0	463.0	453.6	33.3	5.8
MSU	575.0	559.0	570.0	568.0	563.0	567.0	38.5	6.2
Perdita	580.0	581.0	573.0	565.0	590.0	577.8	87.7	9.4

APPENDIX TABLE 2. Perloline concentration of plants sampled July 4, 1979.

Varieties	Replicates Perloline Concentration µg/gm Dry Matter					$\bar{X}$ Reps	Variance	Std. Dev.
	1	2	3	4	5			
Prior (PR x MF)	20.6	23.0	22.0	19.0	20.6	21.0	2.3	1.5
Wendelmoed	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Senu	0.0	0.0	1.0	2.0	2.0	1.0	1.0	1.0
Belimo	10.0	13.0	11.0	8.0	10.5	10.5	3.2	1.8
Barkas	26.8	28,0	30.0	24.9	25.0	26.9	4.6	2.1
Svalof (Sena)	26.8	24.6	27.9	26.2	27.0	26.5	1.5	1.2
Barbarossa	10.0	10.0	13.0	8.0	7.9	9.8	4.3	2.1
Trader	20.6	21.0	19.5	20.0	22.0	20.6	0.9	0.9
AFU	10.0	9.0	9.0	11.0	13.0	10.4	2.8	1.6
Cykado	32.8	30.0	31.3	34.0	33.6	32.3	2.8	1.7
Bundy	10.0	9.5	13.0	8.0	10.2	10.1	3.3	1.8
Bottnia II	0.0	1.0	1.0	0.0	1.0	0.6	0.3	0.5
Mimer	81.5	83.0	80.9	81.0	79.8	81.2	1.4	1.0
Bergamo	10.0	10.0	12.0	9.0	9.7	10.1	1.2	1.1
Dufa	38.8	37.4	39.1	36.0	37.0	37.6	1.6	1.2
Elmet (IR x MF)	87.5	87.9	89.0	85.3	86.0	87.1	2.2	1.4
Mana	26.8	24.6	25.9	27.3	26.8	26.2	1.1	1.0
Cosmos	10.0	11.0	9.0	9.6	10.0	9.9	0.5	0.7
Vagones	210.0	213.0	216.0	207.0	212.0	211.6	11.3	3.4
Tammisto	26.8	27.2	27.0	25.9	26.5	26.6	0.3	0.5
Garanta	10.0	9.5	12.0	8.9	9.3	9.9	1.2	1.0
Loken	26.8	26.8	27.2	33.0	25.0	27.8	9.3	3.1
Festina	26.8	25.1	28.2	26.3	25.4	26.4	1.5	1.2
Paavo	10.0	9.0	11.2	8.3	9.5	9.6	1.2	1.1
Rossa (Hay type)	26.8	30.0	25.0	27.9	26.0	27.1	3.6	1.9
Largo	32.8	33.0	30.5	34.0	29.0	31.8	4.2	2.0
Aberystwyth S53	38.8	38.0	37.5	41.0	36.0	38.2	3.4	1.8
MF-001 (Landis)	26.8	26.8	29.0	27.0	24.0	26.7	3.2	1.8
Angsvingel	26.8	27.0	25.9	30.0	26.0	27.1	2.8	1.6
Janetzki	26.8	27.3	31.0	24.2	25.0	26.8	7.0	2.6
Sequana	10.0	10.0	11.0	8.0	9.0	9.6	1.3	1.1
Merbeem (R.V.P.)	210.0	211.0	207.0	208.0	210.0	209.2	2.7	1.6
Ensign	38.8	41.0	37.0	38.0	35.3	38.0	4.4	2.1
Havingull	300.0	301.0	295.0	299.0	304.0	299.8	10.7	3.3
MSU	215.0	213.0	212.9	221.0	219.0	216.2	13.4	3.6
Perdita	57.0	53.0	55.0	57.0	60.0	56.4	6.8	2.6

APPENDIX TABLE 3. Perloline concentration of plants sampled July 4, 1979.

Varieties	Replicates Perloline Concentration µg/g Dry Matter					$\bar{X}$ Reps	Variance	Std. Dev.
	1	2	3	4	5			
Prior (PR x MF)	197.0	193.7	199.0	195.0	192.0	195.3	7.5	2.7
Wendelmoed	665.0	665.0	663.0	659.0	670.0	664.4	15.8	3.9
Senu	405.0	407.0	405.0	413.0	401.0	460.2	19.2	4.4
Belimo	222.0	220.0	223.0	219.0	221.0	221.0	2.5	1.6
Barkas	758.0	751.0	756.0	758.0	759.5	756.5	11.0	3.3
Svalof (Sena)	513.0	499.0	511.0	509.0	517.0	509.8	45.2	6.7
Barbarossa	330.0	327.0	329.0	330.0	333.0	329.8	4.7	2.2
Trader	360.0	365.0	359.0	360.0	355.0	359.8	12.7	3.6
AFU	818.0	815.0	817.0	811.0	813.7	814.9	7.6	2.8
Cykado	1665.0	1664.5	1667.2	1665.0	1660.0	1664.3	6.9	2.6
Bundy	563.0	561.5	559.0	567.0	550.0	560.1	40.3	6.3
Bottnia II	375.0	373.0	377.0	375.0	371.0	374.2	5.2	2.2
Mimer	952.0	957.0	951.0	952.0	956.0	953.6	7.3	2.7
Bergama	197.0	195.0	197.8	193.0	200.0	196.6	7.2	2.6
Dufa	210.0	209.0	211.0	213.0	210.0	210.6	2.3	1.5
Elmet (IR x MF)	179.0	179.0	175.0	173.0	182.0	177.6	12.8	3.6
Mana	525.0	525.0	521.0	529.0	523.0	524.6	8.8	2.9
Cosmos II	508.0	508.0	506.3	512.0	515.0	509.8	12.6	3.6
Vagones	1255.0	1257.0	1253.0	1251.0	1260.0	1255.2	12.2	3.5
Tammisto	2700.0	2709.0	2713.0	2697.0	2700.0	2703.8	46.7	6.8
Garanta	2875.0	2877.0	2873.0	2871.0	2880.0	2875.2	12.2	3.5
Loken	210.0	211.0	208.0	210.0	212.0	210.2	2.2	1.4
Festina	1305.0	1308.0	1305.0	1313.0	1302.0	1306.6	17.3	4.2
Paavo	142.5	144.0	147.0	142.5	141.0	143.4	5.2	2.3
Rossa (Hay type)	1670.0	1670.0	1675.0	1665.0	1681.0	1672.2	36.7	6.1
Largo	2220.0	2223.0	2221.0	2224.0	2220.0	2221.6	3.3	1.8
Aberystwyth S53	500.0	501.0	497.0	499.0	500.5	499.5	2.5	1.5
MF-001 (Landis)	3700.0	3704.0	3712.0	3711.0	3689.0	3703.2	87.7	9.4
Angsvingel	855.0	856.0	853.9	866.0	851.0	855.2	10.8	3.3
Janetzki	695.0	691.0	693.0	700.0	692.9	694.4	11.9	3.4
Sequana	2098.0	2099.0	2090.0	2093.0	2098.0	2095.6	15.3	3.9
Merbeem (R.V.P.)	1488.0	1488.0	1489.0	1483.0	1493.0	1488.2	12.7	3.6
Ensign	3152.0	3150.0	3149.0	3153.0	3152.0	3151.2	2.7	1.6
Havingull	2280.0	2280.0	2281.0	2283.0	2277.0	2780.2	4.7	2.2
MSU	545.0	545.0	541.0	549.0	543.0	544.6	8.8	2.9
Perdita	3250.0	3255.0	3253.0	3247.0	3245.0	3250.0	17.0	4.1

APPENDIX TABLE 4. Perloline concentration of plants sampled July 18, 1979.

Varieties	Replicates Perloline Concentration µg/gm Dry Matter					$\bar{X}$ Reps	Variance	Std. Dev.
	1	2	3	4	5			
Prior (PR x MF)	275.0	277.0	279.0	280.0	273.0	276.8	8.2	2.8
Wendelmoed	878.0	873.0	879.0	880.0	877.0	877.4	7.3	2.7
Senu	360.0	362.0	365.0	359.0	363.0	361.8	5.7	2.4
Belimo	240.0	242.0	243.0	239.0	247.0	242.2	9.7	3.1
Barkas	788.0	783.0	785.0	787.0	791.0	786.8	9.2	3.0
Svalof (Sena)	678.0	677.0	679.0	671.0	685.0	678.0	25.0	5.0
Barbarossa	525.0	527.0	531.0	523.0	526.0	526.4	8.8	2.9
Trader	166.0	169.0	168.0	159.0	163.0	165.0	16.5	4.1
AFU	1005.0	1007.0	1010.0	1003.0	1005.0	1006.0	7.0	2.6
Cykado	1123.0	1125.0	1129.0	1125.0	1123.0	1125.0	6.0	2.4
Bundy	695.0	695.0	693.0	697.0	691.0	694.2	5.2	2.2
Bottnia II	635.0	633.0	632.9	631.0	641.0	634.6	14.8	3.8
Mimer	1792.0	1791.0	1787.0	1790.0	1792.0	1790.4	4.3	2.1
Bergamo	148.5	147.0	149.0	153.0	145.0	148.5	8.8	2.9
Dufa	216.0	219.0	216.0	213.0	215.0	215.8	4.7	2.2
Elmet (IR x MF)	452.0	451.0	449.0	452.0	457.0	452.2	8.7	2.9
Mana	2042.0	2040.0	2043.5	2039.0	2042.0	2041.3	3.2	1.8
Cosmos II	154.0	156.0	153.0	150.0	147.0	152.0	12.5	3.5
Vagones	1152.0	1159.0	1163.0	1147.0	1149.0	1154.0	46.0	6.8
Tammisto	2750.0	2753.0	2755.0	2747.0	2755.0	2752.0	12.0	3.4
Garanta	1915.0	1919.0	1914.0	1913.0	1909.0	1914.0	13.0	3.6
Loken	360.0	360.0	357.0	363.0	359.0	359.8	4.7	2.2
Festina	605.0	607.0	603.0	601.0	610.0	605.2	12.2	3.5
Paavo	268.0	261.0	267.0	263.0	275.0	266.8	29.2	5.4
Rossa (Hay type)	2700.0	2709.0	2710.0	2695.0	2701.0	2703.0	40.5	6.4
Largo	1215.0	1215.0	1213.0	1217.0	1211.0	1214.2	5.2	2.3
Aberystwyth S53	482.0	483.0	479.0	481.0	489.0	482.8	14.2	3.8
MF-001 (Landis)	6050.0	6053.0	6051.0	6047.0	6055.0	6051.2	9.2	3.0
Angsvingel	940.0	943.0	937.8	932.0	947.0	940.0	31.7	5.6
Janetzki	1700.0	1700.0	1705.0	1701.0	1693.0	1699.8	18.7	4.3
Sequana	2700.0	2707.0	2701.0	2700.0	2695.0	2700.6	18.3	4.2
Merbeem (R.V.P.)	1975.0	1977.0	1979.0	1971.0	1970.6	1974.5	13.6	3.6
Ensign	4000.0	4001.0	3995.0	3996.9	4000.0	3998.6	6.4	2.5
Havingull	1488.0	1489.0	1492.0	1483.0	1490.0	1488.4	11.3	3.4
MSU	2035.0	2037.0	2035.0	2033.0	2032.0	2034.4	3.8	1.9
Perdita	5925.0	5923.0	5925.0	2921.0	5929.0	5924.6	8.8	2.9

APPENDIX TABLE 5. Perloline concentration of plants sampled August 7, 1979.

Varieties	Replicates Perloline Concentration µg/gm Dry Matter					$\bar{X}$ Reps	Variance	Std. Dev.
	1	2	3	4	5			
Prior (PR x MF)	26.8	27.3	26.8	25.4	29.0	27.0	1.6	1.3
Wendelmoed	26.8	24.7	26.8	29.2	25.3	26.6	3.0	1.7
Senu	26.8	25.4	25.4	26.8	27.2	26.3	0.7	0.8
Belimo	26.8	27.5	26.8	24.7	27.0	26.6	1.2	1.1
Barkas	26.8	27.3	26.8	28.2	25.3	26.8	1.1	1.0
Svalof (Sena)	216.0	220.0	217.0	216.0	213.0	216.4	6.3	2.5
Barbarossa	26.8	28.3	27.2	26.8	24.5	26.7	1.9	1.4
Trader	26.8	25.0	30.0	29.3	24.0	27.0	6.8	2.6
AFU	330.0	335.0	327.0	331.0	329.0	330.4	8.8	2.9
Cykado	26.8	30.0	31.3	27.2	23.6	27.8	9.0	3.0
Bundy	26.8	24.7	25.4	27.0	29.2	26.6	3.0	1.7
Bottnia II	26.8	26.8	27.3	24.9	29.1	27.0	2.2	1.5
Mimer	154.0	155.0	153.0	150.0	152.0	152.8	3.7	1.9
Bergamo	26.8	33.0	27.2	26.8	26.5	28.0	7.6	2.8
Dufa	57.0	59.0	53.0	55.0	56.0	56.0	5.0	2.2
Elmet (IR x MF)	26.8	27.9	25.6	25.0	25.7	26.2	1.3	1.2
Mana	282.0	277.0	281.0	283.0	280.0	280.6	5.3	2.3
Cosmos II	26.8	26.8	25.1	27.2	29.0	27.0	1.9	1.4
Vagones	300.0	297.0	305.0	300.0	301.0	300.6	8.3	2.8
Tammisto	788.0	785.0	789.0	783.0	782.0	785.4	9.3	3.0
Garanta	63.2	65.0	61.0	59.0	60.0	61.6	5.9	2.4
Loken	26.8	25.3	24.0	27.0	25.3	25.6	1.5	1.2
Festina	210.0	213.0	211.0	210.0	213.0	211.4	2.3	1.5
Paavo	26.8	25.0	24.9	26.8	24.9	25.6	1.0	1.0
Rossa (Hay type)	179.0	181.0	175.0	182.0	183.0	180.0	1.0	3.2
Largo	154.0	156.0	151.0	151.0	153.0	153.0	4.5	2.1
Aberystwyth S53	26.8	26.8	27.2	25.0	26.9	26.5	0.8	0.9
MF-001 (Landis)	216.0	216.0	212.0	215.0	213.0	214.4	3.3	1.8
Angsvingel	26.8	27.9	25.1	23.2	26.8	26.0	3.4	1.8
Janetzki	360.0	361.0	367.0	359.0	355.0	360.4	18.8	4.3
Sequana	390.0	392.0	397.0	385.0	389.0	390.6	19.3	4.4
Merbeem (R.V.P.)	258.0	260.0	262.0	261.0	257.0	259.6	4.3	2.1
Ensign	407.0	410.0	409.0	395.0	406.0	405.4	36.3	6.0
Havingull	520.0	523.0	527.0	521.0	517.0	521.6	13.8	3.7
MSU	605.0	605.0	510.0	600.0	609.0	605.8	15.7	3.9
Perdita	758.0	758.0	753.0	757.0	759.0	757.0	5.5	2.3