Agronomic potential of sainfoin (Onobrychis viciaefolia) for Montana
by John L Holden

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Agronomy
Montana State University

Abstract:
A study was initiated to evaluate the potential of sainfoin for production in Montana.

A search of the literature revealed sainfoin to be a deep-rooted, long-lived, tetrapoid, perennial legume particularly adapted to dry, well-drained calcareous soils. It is reported not to cause bloat and outyield-ed all other legumes at the Montana Experiment Station when harvested for only one cutting of hay.

Research was undertaken to evaluate the seedling emergence as affected by seeding depth, the protein quality of the seed, and the comparative seasonal chemical composition of sainfoin to other legumes including estrogenic activity.

The experiment on seeding depth indicated that sainfoin will allow a higher percentage of seedling emergence than alfalfa from a depth of three inches or less.

The protein quality of sainfoin seed is similar to that of soybean oil meal and warrants consideration as a source of supplemental protein for livestock feeding.

The seasonal protein content of sainfoin was found to decrease with advancing maturity and dry matter percentage increases. A high negative correlation (-.89) was calculated for protein as related to dry matter percentage in legumes which could lead to a quick, comparative method of calculating crude protein content of legume forages. Sainfoin matures at about the same rate as alfalfa. It has a higher leaf to stem ratio than alfalfa and is lower in crude fiber and protein. Sainfoin has a high percentage of nitrogen free extract in comparison to other legumes which, coupled to its lower protein content, makes it worthy of consideration as a silage crop.

All three stages of red clover and the regrowth stage of alfalfa produced a significantly higher estrogenic response than any of the other legumes. Sainfoin did not exhibit a measurable amount of estrogenic activity at any stage of growth.

Sainfoin possesses potential for both forage and send production in Montana, especially in dryland areas where one cutting of hay is harvested, or on irrigated land where a high-yielding silage crop is desired.

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ABSTRACT

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INTRODUCTION

Dr. R. G. Stapledon, famous British grass specialist, has said, "No grassland is worthy of the name and hardly worth bothering with unless a legume is at work. Find or breed the right legume for every corner of the world and you have developed good grassland in every corner of the world."

The "right" legume for Montana would combine the characteristics of high nutrient production, non-bloating, palatability, competitive ability, seedling vigor, ample seed production, long life, disease and drought resistance, winter hardiness, and adaptability to our soils. Our present legumes have some of these attributes but not all. Birdsfoot trefoil is long-lived, semi-winterhardy, sometimes high yielding, somewhat drought resistant, alkali tolerant and has been reported not to cause bloat. However, it is very slow to establish, may be somewhat lacking in palatability, and will not withstand grazing under drought conditions. Red clover and alsike clover are short-lived and not drought resistant. White clover is low yielding and its close relative, Ladino, lacks winterhardiness, and has high moisture and fertility requirements. The sweetclovers are biennial, susceptible to the sweetclover weevil and contain the undesirable compound coumarin. Alfalfa is good from almost all standpoints except that it may represent a serious bloat hazard in pastures.

Sainfoin (*Onobrychis viciaefolia*) is a deep-rooted, long-lived, tetrapoid, perennial legume introduced from Europe which comes close to possessing all the desired characteristics of a legume for Montana. It is particularly adapted to dry calcareous soils. Piper and Mansfield state, "that sainfoin has never been known to cause bloat." Its common name sainfoin, French for "healthy hay", and the Latin species name Onobrychis, meaning "that for which asses bray", may aptly describe its forage qualities.

These factors were thought to make sainfoin worthy of more detailed evaluation as a possible legume for production in Montana.

LITERATURE REVIEW

The sainfoin plant consists of 3 to 32 stout, erect stems which arise from a branched crown (40). "The leaves are odd-pinnate with 13 to 15 leaflets. The rose colored (rarely white) flowers are in an erect, close raceme 2 to 5 inches long. The roots are reported to reach a diameter of 2 inches and extend to a depth of 20 feet or more. The culture of sainfoin probably dates back about 400 years. It was first cultivated in France; the first definite record according to Vianne was in 1582."(34) At present sainfoin is commonly grown in parts of England and France. Sainfoin (Onobrychis altissima) comes from the Near Eastern Center of Origin. Several species occur wild in that area and in the southern half of Europe and extend eastward toward Russia where extensive research is being done on sainfoin for forage (25), (34).

In England, information obtained from production of sainfoin hay, which is highly prized by racing stables for feeding brood mares and colts, has led to its use on much dry calcareous land, which previously had been nearly valueless (34).

Numerous popular articles have been published in the United States on sainfoin's value as a bee forage (1), (11), (24), (25), (31) and potential for livestock utilization (3) but very little sainfoin is being grown at present.

The two varieties of sainfoin grown in England, common and giant, may have possibilities for production in Montana. They are particularly well adapted to dry, well-drained calcareous soils with a low water table

(26). Pellet, (31), reported them extremely drought and cold resistant. Common differs from giant sainfoin in having finer stems, a more prostrate growth habit, greater persistency of stand, and inability to flower twice a season (18). The first cutting of giant is usually taken for hay and the second left for seed production (3). Giant is usually seeded as a short rotation crop, 2 to 3 years, and common as a long rotation crop, 4 to 7 years. After 7 or 8 years of sainfoin production, good soils become sick in a manner annalogous to "clover sick" soils and must be reseeded to another crop; however, this doesn't occur on the dryer, less fertile soils (34). Eslick (15) reported a stand at Hall, Montana, which has been in existence since 1898. Both giant and common sainfoin are reported to be similar to alfalfa in their ability to withstand frequent close grazing or clipping (16).

Sainfoin is adaptable to good irrigated soils (34). Research by Cooper (10) at the Montana Agricultural Experiment Station showed that sainfoin outyielded all other legume species when compared with first cutting hay yields.

The nutritive content of a plant, within limits, is the final determining factor in its value as a forage crop. Norton (29) states, "There are two factors which greatly influence the feeding value of alfalfa hay; namely, the proportion of leaves to stems and the crude protein content." Morrison's Feeds and Feeding (27) doesn't list the chemical composition of sainfoin. Baker (4), in England, found that like most forages, sainfoin has a lower crude protein content, and a higher crude fiber content as the plant advances toward maturity. The mineral content was found to be somewhat variable. The ratio of leaf dry matter to total dry matter decreases with maturity of the plant (4). The leaf composition is fairly constant irrespective of growth stage, number of cuttings and season; with leaf fiber content being remarkably constant in contrast to that of stem, where it increases with maturity of the plant. The leaf contains more crude protein, ether extract, and mineral matter, particularly calcium, than the stem. (4) Changes which occur in the composition of sainfoin are postulated to be due to variation in stem composition and leaf-stem ratio. Sainfoin is not considered to be a particularly rich source of beta-carotene (4). Baker (4) lists the following as the average composition of 35 samples of sainfoin hay grown in England. For comparison, data on alfalfa from Morrison (27) is given.

Composition of Dry Matter

Constituent	Sainfoin %	Alfalfa %
Moisture	0.0	9.5
Crude Protein	15.0	15.3
Ether Extract	1.8	1.9
Crude Fiber	30.8	28.6
N. Free Extract	45.5	36.7
Total Ash	6.9	8.0
Silica	0.8	-----
Calcium	1.84	1.47
Phosphorous	0.63	0.24
Potassium	1.52	1.97
Chlorides	0.53	-----

Norton (29) reports 40.4 as the average leaf percentage of first cutting alfalfa hay in comparison to 32.3 as the average leaf percentage of sainfoin hay given by Baker (4).

In recent years the estrogenic activity of forages has received consideration in evaluating forages.

The term "estrogen" can be defined as any compound or substance which is capable of causing sexual development in the female, including changes in the vaginal epithelium, hypertrophy of the uterus and mammary glands, and the development of female secondary sex characteristics (44). Estrogenic substances were first discovered in plants in 1926 by Loewe (38). Since that time approximately 50 species of plants have been shown to possess varying degrees of estrogenic activity (32). Interest in forage estrogens was renewed in 1941 when widespread breeding disorders of sheep in Western Australia were found to be caused by the high estrogenic content of subterranean clover (Trifolium subterranean). Since that time, other forage plants which have been found to possess estrogenic compounds of varying activity are red clover (Trifolium pratense), alfalfa (Medicago sativa), white clover (Trifolium repens), strawberry clover (Trifolium frageferum), Ladino clover (Trifolium repens, latum), and birdsfoot trefoil (Lotus corniculatus) (38). Soybean oil meal is also reported to contain estrogenic compounds (38). Red clover is most consistently reported as having estrogenic activity at all stages of growth. It has caused breeding disorders in sheep in Ohio and Oregon (9). Alfalfa varieties are reported to have wide differences in estrogen content (32). Youngman (44) reports Ladak alfalfa in Washington to have increasing estrogenic activity with maturity. Hay samples of alfalfa, alsike clover, white clover and red clover have been reported to possess estrogenic activity (38). Alfalfa silage has been found to possess significantly greater estrogenic activity than fresh alfalfa or alfalfa hay (32), (44). White clover, birdsfoot trefoil, and alfalfa have been reported to be highest in estrogen content during early spring growth (38). The estrogenic activity of forages seems to vary with variety, stage of growth, number of cuttings and environment (32), (44).

Various conflicting theories exist as to the site of production of estrogens in plants. Estrogens occur earliest in the chloroplast fraction, so this would seem the most likely site of production (23).

The estrogenic compounds which have been isolated and identified are the flavinoids, genistein, its glycoside genisten, diadzein, its glycoside diadzin, formononetin, biochanin A and a coumarin-like compound, coumestrol. These all have similar chemical structures which differ mainly in number and location of hydroxyl and methoxyl groups (38). Coumestrol, which has been isolated in all legumes tested, possesses a higher estrogenic activity than any of the other compounds (9).

Quantitative and qualitative determination of estrogens in plant and animal material can be accomplished by chemical methods and bio-assay procedures. Chemical procedures are time consuming, especially with plant materials, since chromatrography and accurate analytical methods are necessary. When the chemical and physical properties of estrogens are not desired, a gross quantitative estimate of the estrogenic activity can be obtained by use of bio-assay techniques (38). With forages, this involves extraction of the estrogen with a solvent and then adding the extract to a control ration which is fed to immature female mice or rats. There is no difference in reliability of results, but mice are less expensive. The animals are fed from three to ten days and then sacrificed. The resultant uterine weight increase is used as a measure of estrogenic activity. The results are usually stated as equivalent to the amount of diethylstilbestrol which would cause the same uterine response (38). Bickoff et al. (8) found that acetone, alcohol, or a 2:1 benzene-alcohol mixture are equally effective in extracting estrogenic compounds from fresh or dried Ladino clover. Drying a forage can cause up to a 75% loss in estrogenic activity. Estrogenic compounds are fairly stable after extraction (9).

In addition to affecting reproduction, plant estrogens have been observed to affect the composition and production of milk and to stimulate growth in castrated male animals (9). Bickoff (9) states, "It now appears that we may need two separate types of forages. One of these would be a low-estrogen forage for breeding stock, because the naturally-occurring estrogens are not desirable here. The second type would be a high-estrogen forage for fattening steers, wethers and poultry." Data has not been published concerning the estrogenic activity of sainfoin or sainfoin seed.

Efficient production of legume forages is dependent upon successful stand establishment. Among factors influencing establishment are seed size, seed viability, rate of seeding, depth of seeding and variety.

Sainfoin seed is produced by the plant in one-seeded pods which are described by Piper (34) as being "brown, indehiscent, lenticular, and reticulated on the surface." Actually, the pod is bean-shaped and bilaterally compressed. It has a rough net veined appearance, and is sometimes referred to as a "cockshead" when it has spines protruding from the ventral edge or keel. Evidence indicates that the spined characteristic is controlled by one pair of genes with spiny dominant to spineless (40).

Sainfoin grown for seed production may produce 25 to 30 bushels of seed in the pod per acre (3). Like alfalfa, it is entomophalous, requiring insect cross-pollination for maximum seed set (15). The podded seed weighs about 30 pounds per bushel, of which one-third, by weight, is pod and shriveled seeds (40). Sainfoin seed remains viable under high drying temperatures after harvest. It is safe to store at 12% moisture and is reported in England to lose its viability after three years storage (30). Sainfoin seed is sold commercially in two forms, milled and unmilled. Milled seed has the pod removed and is kidney-shaped, varying from yellowish green to dark brown in color. Sainfoin seed in the pod weighs 23 grams per 1000 and milled seed weighs 15 grams per 1000 (39). In comparison alfalfa seed weighs 2 grams per 1000 (37). Germination is more uniform from milled seed due to reduction of hard seeds during milling and removal of the seed pod which has been proved to provide mechanical resistance to radicle emergence (40). Unmilled seed retains its viability for a longer period (39).

The recommended seeding rate for alfalfa and sainfoin in England is 22 and 40 pounds of milled seed per acre, respectively (30). In Montana the recommended seeding rate for alfalfa is 10 to 12 pounds per acre.

Piper (34) states that sainfoin should be seeded at a depth of one-half inch or more. The most satisfactory seeding depth for small seeded legumes and grasses in this country is 0.5 inch (5). Two inches is the recommended seeding depth for soybeans(28). Erickson (13) found that as depth of seeding increased, the advantage of large seed over small seed in stand establishment of alfalfa became increasingly apparent. This would cause one to wonder just what the optimum seeding depth for sainfoin might be. Sainfoin seed requires inoculation for proper nitrogen fixation and growth on soils which had not previously grown sainfoin (1). Piper (34) recommends spring seeding, since fall seedings are more apt to winterkill.

Woodman (42) gives the chemical composition of sainfoin seed as follows:

	Unmilled seed	Milled seed
	%	%
Moisture	12.01	8.98
Crude Protein	26.38	36.63
Ether Extract	5.96	7.28
N. Free Extract	33.74	34.06
Crude Fiber	17.86	9.46
Ash	4.05	3.58
Calcium	1.02	.24
Phosphorous	.91	1.15
Chlorides	.11	.11

For a long time protein has been the most expensive ingredient and a limiting factor of livestock rations. Soybean oil meal is commonly used as a protein supplement in livestock rations, including dog and cat foods, because of its high protein content and essential amino acid balance in relation to cereal grains. Recent research indicates that the limiting amino acid for soybean oil meal is methionine, but even without the addition of methionine, it is one of the best quality plant proteins available for livestock feeding (2). Soybean oil meal is listed by Morrison (27) as being about 44 % crude protein. Milled sainfoin seed compares favorably to soybean oil meal in protein percentage, and might be considered for commercial production if it was equal to soybean oil meal in amino acid balance. Soybean oil meal sells commercially at the present time for $83 a ton. Woodman (42) indicated that lambs did very well on unmilled sainfoin seed as a protein supplement and would have equaled gains of lambs on lucerne and clover seed if the pod had been removed.

The search of literature on sainfoin indicated that information was lacking in the areas of recommended seeding depth, protein quality of the seed, and comparative seasonal chemical composition including estrogenic activity. Research was undertaken to evaluate these qualities of sainfoin.

SEEDING DEPTH

Materials and Methods

Because of the exceptionally large size of sainfoin seed and lack of information regarding proper seeding depth, an experiment was designed to evaluate the effect of seeding depth on the resulting seedling emergence of sainfoin.

One hundred alfalfa seeds and an equal number of sainfoin seeds were planted at eight different depths, starting at one-half inch and increasing in one-half inch increments to four inches, in soil on a bench in the greenhouse. A split-plot experimental design was used with seeding depths assigned at random to the main plots and species to subplots. Three replications were planted. Germination percentage of the seed was obtained before planting. Relative rate of emergence was noted and results were compiled as percentage of viable seed emerging. Alfalfa was included in the experiment as a check.

Results and Discussion

The germination percentage of sainfoin seed and alfalfa seed was 78 and 95 respectively. The sainfoin seed had been stored for three years which refutes data by Owen (30) that indicated sainfoin seed loses its viability during that period of time.

The alfalfa seedlings emerged a day earlier than the sainfoin seedlings at depths of seeding to two inches. Sainfoin at greater depths had the faster emergence. The sainfoin seedlings emerged with the hypocotyl pushing the cotyledons straight up through the soil as did alfalfa. This

Table I. Percentage of viable sainfoin and alfalfa seeds which germinated and emerged from various seeding depths. 1/

Seeding depth in inches	Percent age of sainfoin seeds emerging	Percent age of alfalfa seeds emerging
.5	88.5 a b	60.2 a
1.0	97.0 a	57.9 a
1.5	76.9 a b	56.3 a
2.0	88.9 a b	37.2 a b
2.5	63.7 b c	22.8 b c
3.0	38.9 c	17.6 b c
3.5	9.4 d	1.1 c
4.0	1.3 d	1.4 c

1/ Values underlined within a seeding depth are significantly different at P = .05.
Values within the legume followed by the same letter are not significantly different at P = .05 (12).

is different from some legumes, such as beans, in which the hypocotyl is sharply bent and pulls the cotyledons to the soil surface.

The results of the experiment are shown in Table I as percentage of viable seeds which emerged from the soil as seedlings.

Alfalfa had an emergence of 60.2 % from the 0.5 inch seeding depth which is comparable to field emergence. Sainfoin exhibited good emergence from the first four seeding depths, fair emergence in the 2.5 and 3 inch seeding depths and poor in the 3.5 and 4 inch depths. In comparison, alfalfa had good emergence in the first three seeding depths, fair emergence in the 2, 2.5 and 3 inch seeding depths and poor in the 3.5 and 4 inch depths.

The experiment was designed to place maximum precision in statistical analysis on the seeding depth by legume interaction. A significant difference greater than P = .05 existed between legumes. Seeding depth differences were significant at P = .01 with the interaction having significance greater than P = .05.

This experiment indicates that sainfoin is superior to alfalfa in seedling emergence and will produce a greater percentage of seedlings than alfalfa from a seeding depth of 3 inches or less.

PROTEIN QUALITY OF SAINFOIN SEED

Materials and Methods

The experiment herein described was designed to compare the protein quality (essential amino acid balance) of sainfoin seed (Onobrychis viciaefolia) and pigweed seed (Amaranthus retroflexus) with that of soybean oil meal (Glycine max) and to measure its effect on rat growth and feed consumption.

Fifteen three-week-old female white rats were divided into three equal weight groups of five rats each.

Rations were formulated to contain 10% crude protein, on the basis of work by Rama Rao et al. (35) which indicated that the minimum rat requirement of protein for maximum nitrogen retention is 10% and the minimum requirement for maximum growth is 8.8%. Crude protein contents of unhulled sainfoin seed, soybean oil meal and pigweed seed used in the rations were 24.6%, 42.0% and 15.0% respectively.

Table II shows the rations used for the experiment.

Table II. Composition of rations used to evaluate protein quality of sainfoin seed meal.

Ingredients	Pigweed seed meal ration %	Sainfoin seed meal ration %	Soybean oil meal ration %
Vitamin premix	1.0	1.0	1.0
U.S.P. 14 salt mix	4.0	4.0	4.0
Alpha cellulose	5.0	5.0	5.0
Corn oil	10.0	10.0	10.0
Corn Starch	13.3	39.3	56.2
Protein source	67.7	40.7	23.8

Each group of rats was fed ad libitum for 28 days. Weights were taken at about 10 day intervals during the feeding trial and feed consumption was noted.

Results and Discussion

The soybean oil meal ration and sainfoin seed meal ration produced significantly (P = .05) greater rat gains than the pigweed seed meal ration during the last 17 days of the feeding trial (Table III). There was no significant difference between weight gains of rats fed the sainfoin and pigweed rations during the first 11 days of the experiment, nor between weight gains from the sainfoin and soybean rations throughout the trial.

Table III. Average rat weight gains and feed efficiency during a feed trial conducted using pigweed seed meal, sainfoin seed meal and soybean oil meal as protein sources.1/

Treatment	Weights taken in grams Int. wt.	11 day gain	11-21 day gain	21-28 day gain	Final wt.	Total gain	Avg. daily	Grs. feed per 100 grs. gain
Soybean oil meal ration	56.2	38.6a	28.1a	14.2a	136.7	80.5a	2.88a	396
Sainfoin seed meal ration	55.9	31.5ab	30.4a	10.5a	128.3	72.4a	2.59a	460
Pigweed seed meal ration	57.1	20.3b	11.0b	3.3b	91.7	34.6b	1.24b	643

1/ Differences among rations are significant at P = .05 if not followed by the same letter (12).

Kumpta and Harper (22) stated that, "A fall in food intake can usually be detected within a short time after an animal has been fed ad libitum a diet in which an amino acid imbalance has been created." This occurred with the rats on the pigweed seed ration. They ate a total of 1112 grams of feed compared to 1595 grams of feed for the rats on soybean and 1664 grams of feed for the rats on sainfoin. They were also more nervous and irritable than the rats in the other two groups. This agrees with previous research by Young and Dunn (43).

The higher feed consumption and lower feed efficiency of the rats on sainfoin as compared to those on soybean may have been due to the pods on the sainfoin seed which were ground with the seed and mixed as part of the ration. These pods were unpalatable and the rats wasted part of them. The pods are high in fiber (42) and should contain less energy than an equal amount in weight of corn starch which was their counterpart in the soybean oil meal ration.

The author surmises, as did Woodman and Evans (42), that milled sainfoin seed with the pods removed would compare much more favorably to soybean oil meal with respect to protein quality.

In England, sainfoin is reported to produce about 600 pounds of milled seed per acre (3). Compared to the present price of soybean oil meal, this would be worth about $24 per acre. Sainfoin seed could be harvested every year. The value of two years' production of sainfoin seed would be equal to a 24 bushel wheat crop in an alternate year - summer fallow cropping system.

The value of the seed as a protein supplement for the livestock industry plus the forage production of the plant makes sainfoin worthy of consideration for production in Montana.

SEASONAL CHEMICAL COMPOSITION

Materials and Methods

In order to obtain a comparison of sainfoin with other forage legumes, weekly harvests were made from eight legumes and one grass grown in plots located approximately five miles west of Bozeman on the Montana Agricultural Experiment Station, Crops and Soils Field Research Laboratory. The forages compared were sainfoin (Onobrychis viciaefolia), Ladak alfalfa (Medicago sativa), tetrapoid alsike clover (Trifolium hybridium), Kenland red clover (Trifolium pratense L.), Ladino clover (Trifolium repens), Tana birdsfoot trefoil (Lotus corniculatus), cicer milkvetch (Astragulus cicer), sickle milkvetch (Astragulus falcatus) and orchardgrass (Dactylis glomerata).

The harvests were made on Friday afternoon each week. The height of cutting was 2 inches. Height and stage of maturity were noted at time of harvest. The samples were selected at random from the plots, placed in paper bags and oven dried in the forage dryer at about 68° C for three days. Leaf and stem separations were made at the hay stage. Ladino clover was not included in the comparison until August 3, when samples were taken on regrowth from the hay cutting. Dry matter percentage was determined and samples ground through a 1/32 inch screen in a Wiley mill for crude protein determination by the Kjeldahl method. Samples of leaf and stem separation from alfalfa and sainfoin were analyzed by the Chemistry Research Laboratory for crude fiber, ether extract, and total ash. Nitrogen free extract was calculated using the other values.

Results and Discussion

The relative rate of maturity of sainfoin in relation to other legumes and orchardgrass is shown in Table IV. Sainfoin reached the hay stage one day earlier than alfalfa. It did not exhibit leaf loss in comparison to alfalfa which, at Bozeman, normally loses some of its lower leaves before reaching the hay stage. Sainfoin and the two milkvetches were less affected by frost in the fall than the other legumes. Sainfoin was the tallest of all legumes during the development of the first cutting, Table V.

Comparative seasonal variation in dry matter is shown in Table VI. Variations from normal for some dates may be attributed to rain and moisture on the forage when the samples were harvested. Sainfoin did not exhibit as high a dry matter percentage as might be expected considering its height and large, rank growing stems.

The protein content of sickle milkvetch was considerably higher than the other legumes throughout the experiment, Table VII. Sainfoin and alfalfa had a lower protein content than previously reported (4), (27). This could have been due to environmental, variety, or inoculation effects. The trefoil plants were yellow and appeared to be suffering from improper inoculation or a lack of nitrogen. This could account for its rapid decline in protein content.

The protein and dry matter comparisons were not statistically analyzed because just one sample was taken in the field to obtain the data of

Table IV. Growth stages of 8 legumes and orchardgrass on various harvest dates at Bozeman in 1962.1/

Date of Harvest	Alfalfa	Alsike Clover	Birdsfoot Trefoil	Cicer Milk-vetch	Red Clover	Sickle Milk-vetch	Sainfoin	Ladino Clover	Orchard-grass
				First Crop Growth					
5-18	veg. *	veg.	veg.	veg.	veg.	veg.	veg.	---	veg.
5-25	veg.	veg.	veg.	veg.	veg.	veg.	veg.	---	veg.
6-1	veg.	veg.	1st buds	veg.	veg.	veg.	veg.	---	boot
6-8	veg.	veg.	1st bloom	veg.	veg.	1st buds	veg.	---	heading
6-15	1st leaf loss	1st bud	30% bloom	veg.	1st bloom	adv. bud	1st buds	---	heading
6-20	---	---	75% bloom	---	---	---	---	---	pre-bloom
6-22	1st buds	1st bloom	Fullbloom	1st bud	20% bloom	pre-bloom	1st bloom	---	blooming
6-26	---	---	---	---	---	---	50% bloom	---	---
6-27	5% bloom	---	---	---	---	pods form	---	---	blooming
6-29	35% bloom	75% bloom	75% pods	adv.bud	fullbloom	50% pods	75% bloom	---	blooming
7-2	---	fullbloom	---	---	---	---	---	fullbloom	---
7-5	---	---	---	50% bloom	---	---	---	---	---
7-6	65% bloom	15% d.f.*	85% pods	60% bloom	40% d.f.	100% pods	75% d.f.	---	s.f.*
7-14	5% pods	50% d.f.	100% pods	15% pods	80% d.f.	s.f.	90% pods	---	s.f.
7-20	25% pods	75% d.f.	s.f.	75% pods	90% d.f.	s.f.	s.f.	---	s.f.
7-27	50% pods	95% d.f.	s.f.	100% pods	s.f.	s.f.	s.f.	---	s.f.
				Regrowth After Cutting					
8-3	veg.	veg.	25% bloom	veg.	5% bloom	veg.	veg.	10% bloom	veg.
8-10	veg.	5% bloom	40% pods	veg.	15% bloom	veg.	veg.	20% bloom	veg.
8-17	15% bloom	10% bloom	90% pods	veg.	50% bloom	veg.	10% bloom	35% bloom	veg.
8-24	40% bloom	20% bloom	100% pods	veg.	70% bloom	veg.	20% bloom	50% bloom	veg.
8-31	50% bloom	15% d.f.	s.f.	veg.	30% d.f.	veg.	35% bloom	100% bloom	veg.
9-7	50% d.f.	75% d.f.	s.f.	veg.	60% d.f.	veg.	60% d.f.	45% d.f.	veg.
9-14	65% d.f.	95% d.f.	s.f.	veg.	85% d.f.	veg.	90% d.f.	90% d.f.	veg.
9-21	frosted	s.f.	s.f.	veg.	95% d.f.	veg	p.f.*	90% d.f.	veg.

1/ Underscored stages indicate dates and stages considered to be correct for haying; samples for leaf-stem separations were taken on these dates.

* veg. = vegetative stage. d.f. = dried flowers after blooming. s.f. = seeds forming. p.f. = pods forming.

Table V. Height in inches of 8 legumes and orchardgrass on various harvest dates at Bozeman in 1962.[1/]

Date of Harvest	Alfalfa	Alsike Clover	Birdsfoot Trefoil	Cicer Milkvetch	Red Clover	Sickle Milkvetch	Sainfoin	Ladino Clover	Orchard-grass
				First Crop Growth					
	in.	in.	in.	in.	in.	in.	in.	in.	in.
5-8	12	4	5	5	8	6	8	-	12
5-25	16	9	9	7	9	10	12	-	14
6-1	19	12	12	11	15	15	16	-	19
6-8	22	14	14	15	19	20	20	-	24
6-15	25	20	17	16	22	21	23	-	30
6-20	-	-	20	-	-	-	-	-	-
6-22	29	24	21	21	24	27	33	-	44
6-26	-	-	-	-	-	-	34	-	-
6-27	32	-	-	-	-	29	-	-	47
6-29	32	24	22	22	24	30	36	-	48
7-2	-	24	-	-	-	-	-	14	-
7-5	-	-	-	24	-	-	-	-	-
7-6	34	24	24	25	29	33	38	-	48
7-14	36	24	24	26	29	33	38	-	48
7-20	36	24	24	26	29	33	38	-	48
7-27	36	24	24	26	29	33	38	-	48
				Regrowth after cutting					
8-3	14	7	13	2	16	5	9	7	7
8-10	16	9	13	4	20	7	12	10	12
8-17	21	11	13	6	24	9	17	10	13
8-24	22	10	14	8	24	10	18	12	14
8-31	21	10	14	6	24	10	22	11	13
9-7	18	10	13	8	24	10	20	10	13
9-14	18	9	13	8	23	9	20	10	13
9-21	16	9	13	9	23	11	19	9	12

1/ Values underlined are heights at hay stage.

Table VI. Dry-matter content of 8 legumes and orchardgrass on various harvest dates at Bozeman in 1962.[1]

Date of Harvest	Alfalfa	Alsike Clover	Birdsfoot Trefoil	Cicer Milkvetch	Red Clover	Sickle Milkvetch	Sainfoin	Ladino Clover	Orchard-grass
	%	%	%	%	%	%	%	%	%
				First crop growth					
5-18	16.8	17.1	15.5	10.1	18.0	23.7	16.0	----	21.6
5-25	14.8	15.0	14.3	14.4	15.8	17.8	14.3	----	14.7
6-1	15.9	12.9	14.4	13.7	13.7	15.9	14.5	----	19.2
6-8	17.0	13.3	16.1	14.2	14.4	17.6	15.3	----	19.5
6-15	18.9	11.9	16.3	13.7	18.2	18.5	16.4	----	24.3
6-20	----	----	<u>18.5</u>	----	----	----	----	----	----
6-22	23.9	15.5	19.1	16.2	22.9	23.7	20.9	----	29.5
6-26	----	----	----	----	----	----	<u>27.3</u>	----	----
6-27	<u>28.4</u>	----	----	----	----	33.2	----	----	<u>39.1</u>
6-29	31.2	18.2	21.8	20.9	<u>27.2</u>	23.0	29.0	----	36.0
7-2	----	<u>21.9</u>	----	----	----	----	----	23.6	----
7-5	----	----	----	<u>23.8</u>	----	----	----	----	----
7-6	33.2	22.0	23.3	24.9	28.1	26.6	34.4	----	48.2
7-14	31.5	22.5	23.2	27.3	27.3	30.0	37.5	----	38.9
7-20	31.9	24.5	23.6	27.4	30.5	31.3	33.3	----	33.7
7-27	30.3	24.3	25.4	26.3	30.5	42.3	34.7	----	49.1
				Regrowth after cutting					
8-3	21.1	16.0	24.6	22.8	19.3	27.0	20.9	17.4	27.4
8-10	24.4	17.7	25.0	17.2	19.7	23.9	20.0	20.6	24.8
8-17	24.8	18.4	26.3	14.9	21.3	23.0	20.9	16.6	28.9
8-24	29.6	22.7	29.0	22.0	25.5	28.0	26.2	19.5	33.0
8-31	29.3	21.9	23.8	18.3	27.0	25.0	24.0	17.5	29.0
9-7	35.5	27.5	28.3	26.3	27.9	27.3	26.5	24.0	30.9
9-14	38.0	26.7	28.5	24.0	28.4	27.5	29.4	25.9	35.1
9-21	45.0	32.5	29.3	26.6	35.6	31.2	31.2	24.7	32.2

1/ Values underlined are dry matter percentage at hay stage.

Table VII. Crude protein content of 8 legumes and orchardgrass on various harvest dates at Bozeman in 1962.1/

Date of Harvest	Alfalfa	Alsike Clover	Birdsfoot Trefoil	Cicer Milkvetch	Red Clover	Sickle Milkvetch	Sainfoin	Ladino Clover	Orchard-grass
	%	%	%	%	%	%	%	%	%
				First crop growth					
5-18	26.3	22.8	21.9	31.8	21.3	33.3	23.5	----	19.7
5-25	23.5	22.6	18.0	29.6	22.0	30.7	21.9	----	14.9
6-1	20.2	22.8	16.9	22.5	20.0	27.9	18.9	----	12.3
6-8	22.2	21.5	13.4	23.7	20.6	25.0	15.4	----	9.7
6-15	18.6	19.5	12.3	17.6	19.1	20.2	14.9	----	8.8
6-20	----	----	11.5	----	----	----	----	----	----
6-22	14.3	16.7	8.3	14.7	13.4	15.8	13.4	----	7.7
6-26	----	----	----	----	----	----	12.3	----	----
6-27	14.5	----	----	----	----	19.2	----	----	6.9
6-29	8.8	14.9	7.9	14.0	11.4	20.2	9.2	----	8.1
7-2	----	14.6	----	----	----	----	----	----	----
7-5	----	----	----	13.3	----	----	----	----	----
7-6	9.9	15.4	7.0	10.8	12.5	17.6	9.4	----	6.8
7-14	12.9	14.5	6.8	10.3	10.5	16.2	7.7	----	7.0
7-20	11.0	12.9	6.1	11.0	12.1	16.7	9.2	----	9.2
7-27	11.4	13.6	6.1	11.2	12.5	16.7	6.6	----	6.6
				Regrowth after cutting					
8-3	22.4	23.9	9.9	27.4	19.5	31.8	21.9	21.3	16.2
8-10	20.2	22.6	7.5	27.6	14.5	28.5	18.2	20.4	14.7
8-17	18.9	22.6	6.8	25.0	17.3	29.6	15.8	21.5	14.0
8-24	19.7	20.6	8.1	22.2	15.8	27.4	15.8	19.5	12.7
8-31	17.1	20.2	8.6	22.2	16.5	24.6	15.6	18.2	14.0
9-7	17.1	18.0	6.8	19.5	16.5	25.5	13.6	19.5	12.5
9-14	16.5	18.0	6.6	17.6	15.8	25.5	12.1	17.6	11.6
9-21	12.5	15.8	6.4	17.3	14.3	23.5	11.0	16.9	9.9

1/ Values underlined are protein percentages at hay stage.

Tables VI and VII. A correlation coefficient of -.89 was calculated for the relation of protein content to dry matter percentage. All protein values having a corresponding dry matter percentage of 20 or more were used in this analysis. This agrees with previous research which has shown that protein content decreases and dry matter percentage increases with plant maturity (4), (19), (33), (36). The high correlation of protein content to dry matter percentage indicates that the protein content of legume forages might be accurately predicted from dry matter percentage when the dry matter percentage exceeds 20.

Leaf and stem compositions of forages in the hay stage are shown in Table VIII. Ladino clover was not included because it is almost all leaves if the petiole is considered part of the leaf fraction as it was with the other forages. Sickle milkvetch had a higher percentage of leaves than any of the other forages. Milkvetch leaves also contain a higher percentage of total plant protein than any of the other forage leaves. The leaf percentage of sainfoin is higher than indicated in previous research, 43.8 as compared to 32.3 as reported by Baker (4). This might be accounted for by his inclusion of the inflorescence in the stem portion, whereas it was included in the leaf portion in this experiment. The alfalfa leaf percentage was nearly the same as shown in previous research, 39.1 as compared to 40.4 reported by Norton (29).

The nutrient content of sainfoin hay and its leaf and stem separations, as compared to corresponding alfalfa values, is reported in Table IX.

Table VIII. Leaf-Stem comparison of 7 legumes and orchardgrass at hay state at Bozeman in 1962.

	Legume							
	Alfalfa	Alsike Clover	Birdsfoot Trefoil	Cicer Milkvetch	Red Clover	Sickle Milkvetch	Sain-foin	Orchard-grass
Date of harvest	6-27	7-2	6-20	7-5	6-29	6-27	6-26	6-27
Stage of growth	5% Bloom	Full Bloom	75% Bloom	50% Bloom	Full Bloom	Pods Forming	50% Bloom	Bloom Stage
Height	32	24	20	24	24	29	34	48
Leaf protein %	23.7	21.3	18.2	18.6	18.0	26.1	20.2	9.4
Stem protein %	8.6	7.0	6.6	6.6	6.4	9.4	6.1	4.1
Total protein %	14.5	14.6	11.5	13.3	11.4	19.2	12.2	6.9
Leaf dry matter %	27.2	25.3	18.4	23.8	28.1	35.9	28.3	41.5
Stem dry matter %	29.1	18.1	18.6	23.7	26.4	29.4	27.3	36.4
Total dry matter %	28.3	21.9	18.5	23.7	27.2	33.2	27.3	39.1
% leaves by weight	39.1	53.2	42.5	56.1	46.2	58.6	43.8	53.6
% total protein in leaves	63.9	77.6	67.2	78.5	72.9	79.7	72.5	73.0

Table IX. Nutrient composition of leaf and stem separations of alfalfa and sainfoin hays grown at Bozeman in 1962.

	Leaves	
	Alfalfa	Sainfoin
	%	%
Crude Protein	22.9	19.0
Ether Extract	2.9	2.3
Crude Fiber	16.2	15.0
N. Free Extract	41.6	50.4
Total Ash	10.4	6.9
	Stems	
	Alfalfa	Sainfoin
	%	%
Crude Protein	8.6	5.7
Ether Extract	1.1	1.0
Crude Fiber	43.5	34.3
N. Free Extract	35.1	47.9
Total Ash	6.0	5.7
	Total	
	Alfalfa	Sainfoin
	%	%
Crude Protein	14.2	11.5
Ether Extract	1.8	1.6
Crude Fiber	32.8	25.8
N. Free Extract	37.6	49.0
Total Ash	7.7	6.2

Sainfoin had a lower protein and crude fiber content than alfalfa which resulted in a higher nitrogen free extract value for sainfoin. These results agree with those reported by Baker (4) and Morrison (27); and indicate that sainfoin might have possibilities as a silage legume, due to its wider carbohydrate-protein ratio. This also places a higher value on sainfoin hay which evidently contains more nutrients than its large, rough, stemy appearance indicates.

The data obtained comparing the seasonal chemical composition of sainfoin to other legumes grown in Montana indicates that sainfoin may be of similar nutrient value.

ESTROGENIC ACTIVITY

Materials and Methods

The forage species used for this experiment were the same as those used for seasonal chemical composition.

Three harvests were made from the same plots on the experimental farm:

1. Vegetative - harvested at a constant date -- June 20.
2. Hay stage - harvested as specie matured to proper stage for hay.
3. Regrowth stage- harvested at a constant date -- August 15.

The samples were collected in plastic bags and fresh frozen as soon as possible to prevent alteration of estrogenic compounds within the plants. They were stored in a frozen state at approximately -20^0 C. until used for bio-assay in January.

Female mice, 19 to 21 days old were obtained from the Veterinary Research Laboratory and used as the bio-assay animal for determining the estrogenic activity of the forages.

The basic mouse ration consisted of:

1.5% vitamin premix

4.0% U.S.P. 14 salt mix

5.0% alpha cellulose

10.0% corn oil

20.0% milk casein

59.5% corn starch

None of these ingredients were reported to possess estrogenic activity. A pretrial using varying levels of diethylstilbestrol indicated that the maximum variation in initial weights of mice which will produce accurate data is 3 grams. A 7 day feeding period was found to be necessary to obtain a measurable uterine growth from an estrogenic feed. This is in conflict with data by Swierstra (38), which indicated that maximum uterine growth could be obtained in a 3 day period. Strains of mice may differ in their response to estrogenic stimulation and the rate of response must be determined before reliable results can be obtained. This strain of mice required 60 grams of mouse ration to feed five mice 7 days in comparison to 50 grams of control ration used by Bickoff (6) for the same period.

In the following experiments, the mice were weighed to the nearest one-half gram and separated into groups of equal body weight. A two-gram variation in initial weight was allowed in treatment groups. Each treatment was allotted five mice. The mice were selected at random from the desired weight groups and distributed among treatment groups so that the initial weight of each treatment group was as nearly equal as possible. Each group of five mice was housed together in wire laboratory cages for the duration of the six to seven day feeding period. The mice were fed the test diet in equal increments each day until 60 grams of the test diet were consumed by the five mice. Chloroform was used to sacrifice the mice and the freshly excised uteri were trimmed, blotted on tissue paper and weighed on a Roller-Smith balance.

The first group of feed treatments was prepared with increasing levels of a diethylstilbestrol (D.E.S.) to establish a uterine dose response curve for the strain of mice used. A stock solution of diethylstilbestrol was made by mixing 5 mg. of the drug with 500 ml. of 95% ethanol. One ml. of this solution when added to 100 grams of mouse ration gives a 0.1 ug/gr. concentration of diethylstilbestrol.

The stilbestrol rations were prepared by pipetting the desired amount of diethylstilbestrol stock solution into 20 ml. of 95% ethanol and mixing this with 60 grams of mouse ration. The rations were dried in open air at room temperature for 48 hours and then fed to the mice. The mouse rations fed in this trial contained the following micrograms of diethylstilbestrol per gram of ration: .02, .04, .06, .08, and 0.1 and the control ration was treated with plain ethanol.

An experiment was designed to determine if inclusion of actual forage in the ration would produce a uterine response indicating estrogenic activity. The ration consisted of 20 grams of dried ground forage mixed with 40 grams of mouse ration. Three stages (vegetative, hay and regrowth) of alfalfa were fed with two methods of storage. One group of stages was dried when collected and then stored. The other group was dried in February after storage in a frozen state. Sainfoin seed was included in this experiment to see if it would exhibit estrogenic activity in the natural form. This experiment produced non-significant results and demonstrated the need for use of solvent extraction methods.

The first extraction procedure used was a method described by Bickoff et al. (6) for extracting estrogenic compounds from fresh forage.

Fresh frozen forage was ground through the sausage grinder in the Meats Laboratory. A 400-gram portion was removed for moisture determination and 100 grams were extracted. The estrogenic extract was prepared by macerating the 100 grams of ground plant tissue with 350 ml. of acetone in a Waring commercial blender for two minutes. The entire contents of the blender bowl were placed in a Buchner funnel and suction filtered through Whatman No. 1 filter paper. The filter cake was washed with an additional 100 ml. of acetone and the combined filtrate was concentrated over low heat to a volume of about 30 ml. This aqueous concentrate was transferred to a separatory funnel and extracted twice with 50 ml. of ethyl ether. The ether extracts were put in 120 ml. bottles and allowed to evaporate under a hood with a suction fan. The bottles were corked and put in a freezer until addition to the mouse bio-assay ration. The residue inside the bottle was dissolved in 100 ml. of 95% ethanol and added to the mouse ration at the desired level.

An experiment was designed to establish a dose response curve with varying levels of extract from red clover and alfalfa in the vegetative stage. The rations were prepared by adding the desired amount of extract to 20 ml. of 95% ethanol and mixing thoroughly with 60 grams of ration. The mixture was allowed to dry for 48 hours in open air and fed to the mice. Bickoff et al. (6) recommended a level of 8 to 10 grams of forage extract per 100 grams of mouse ration. Levels of extract from 5, 10, 15, 20, 40 and 80 grams of forage per 100 grams of mouse ration were fed. Non-significant results were obtained from this experiment; so a modification of the extraction procedure used by Swierstra (38) was used in subsequent trials, hereafter referred to as the Canadian Method and is described below.

Samples of frozen forage were dried in the forage dryer at 68° C. for three days. Moisture percentages were calculated and the dried samples ground through the 1/32 inch screen in the Wiley mill.

Sixty grams of ground forage were mixed with 200 ml. of acetone and 150 ml. of 95% ethanol. This was refluxed over low heat in a 600 ml. beaker for one hour and suction filtered through Whatman No. 1 paper in a Buchner funnel. The filter cake residue was mixed with 150 ml. of acetone and 150 ml. of 95% ethanol and refluxed for one more hour. This was suction filtered by the same procedure as above. The filtrates were combined and evaporated over low heat to approximately 30 ml. This extract was mixed with 60 grams of mouse ration and dried for 48 hours before being fed to the mice. This method produced measurable results when used in an experiment with three stages of alfalfa. The hay stage of alfalfa which had been stored in a dry form from harvest until extraction was included in the above experiment to check the effect of storage on estrogenic activity.

Extracts of all legume forage stages plus sainfoin seed were mixed into mouse rations. Orchardgrass extract was not included because of a shortage of mice. The rations were divided according to forage growth stages and fed to mice within a two-gram weight group. A diethylstilbestrol control ration of .0067 ug/gr. of mouse ration was fed during this experiment as a check against the previously established diethylstilbestrol response curve.

The hay stages of all nine forages were also tested using the Bickoff et al. extraction method (6). The rations were prepared having a level of 27.75 grams of extract per 100 grams of ration. This gave a comparison of the Bickoff and Canadian procedures for obtaining estrogenic extracts from forages.

The results of these experiments where applicable, were statistically analyzed using Duncan's multiple range test (12). Correlations were determined on the relation of mouse body weight to uterine weight in estrogenically stimulated and unstimulated mice.

Estrogenic activity of forages where applicable was calculated by regression as equivalent to the micrograms of diethylstilbestrol per pound of dry matter required to produce an equal response in the bio-assay animals.

Results and Discussion

A table of results is not presented for the pretrial. However, valuable experience and information for succeeding trials was obtained from it. The range in initial weight of the mice was too wide to give reliable data. Error in formulating levels of diethylstilbestrol for the ration also decreased the accuracy of the experiment. The data did indicate that three grams was the maximum variation in initial weights of mice within an experiment which would give reliable results. Seven days were required for a measurable amount of uterine growth. The mice required 12 grams of feed each for a seven-day feeding period. This knowledge was essential to proper conduction of following trials.

A dose response curve was established using increasing levels of diethylstilbestrol (Figure 1.). The uterine weights increased up to the .06 ug/gr. of control ration level and started to decline in the two higher levels fed (Table X). A correlation was calculated on the relation of initial weight of mice to the resultant uterine weight after treatment. There was no significant relationship of body weight to uterine weight of mice on control rations containing no estrogenic stimulation. A significant positive correlation coefficient of .44 (significant at P = .05) was calculated for relation of body weight to uterine weight of mice on rations containing diethylstilbestrol. The regression line for this is shown in Figure 1. All following data was statistically analyzed using uterine weight as percentage of body weight which was considered the most accurate indication of the estrogenic activity of a treatment.

The experiment having actual ground forage as part of the mouse ration did not produce any significant estrogenic stimulation (Table XI). There could have been an alteration of estrogenic activity due to the difference in methods of drying which had been used on the alfalfa but none occurred. Sainfoin seed was not significantly different in estrogenic

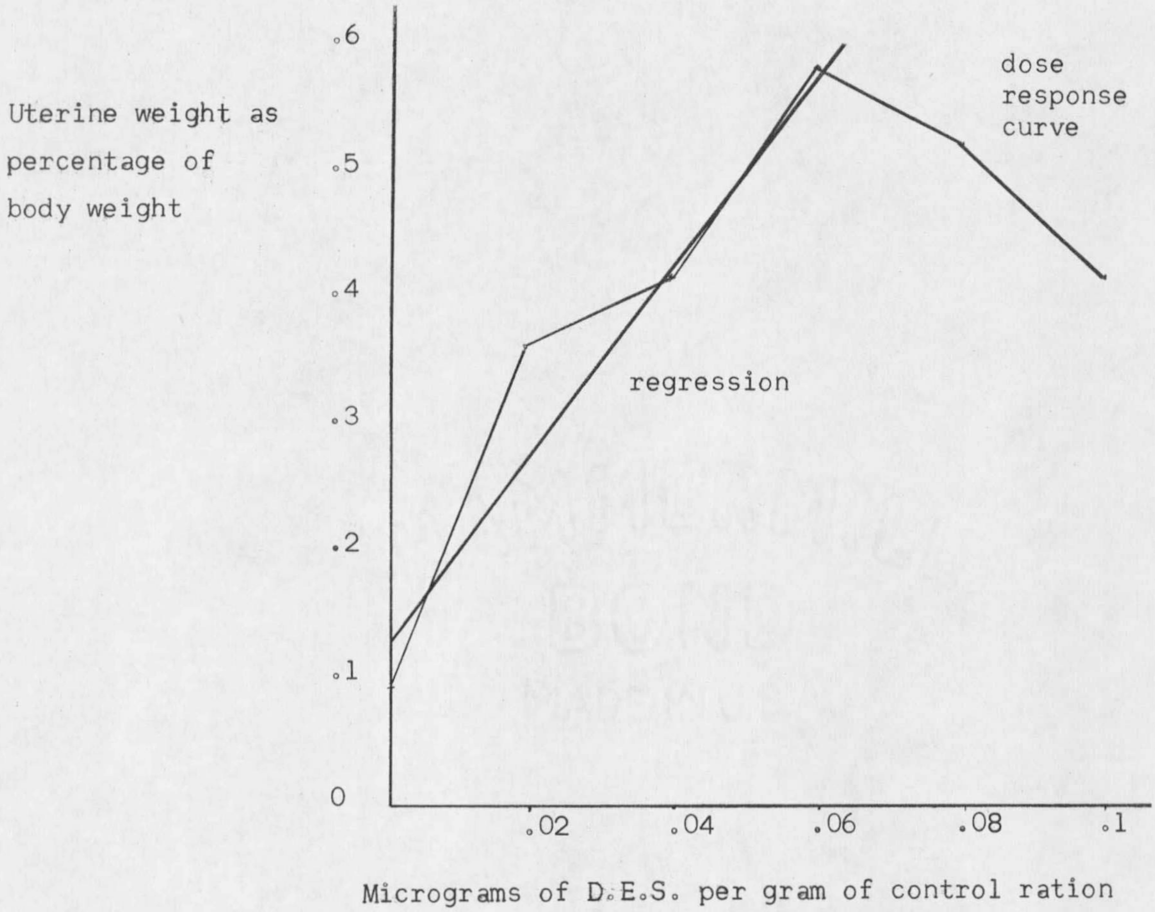

Figure 1. Diethylstilbestrol dose response curve and regression of uterine weight as percentage of body weight on micrograms of diethylstilbestrol in feed at levels of 0 to .06 micrograms per gram of feed.

Table X. Estrogenic activity of various levels of Diethylstilbestrol added to a control ration.

Micrograms D.E.S. per gram of ration	Actual Amount of D.E.S. in feed	No. of mice per Treatment	Average uterine wt. in milligrams	Average uterine weight as percentage of body weight
Control	0	5	11.7	.09740
.02	.12	5	48.42	.34000
.04	.24	5	53.34	.37857
.06	.36	5	81.40	.57115
.08	.48	5	69.08	.54719
.01	.60	5	54.0	.40005

Table XI. Estrogenic activity of three stages of alfalfa with different storage procedures and sainfoin seed when fed without extraction as part of the mouse ration.

Treatment	Actual grams of Dry Matter in Feed	Number of Mice per Treatment	Average Uterine Weight in Milligrams	Average Uterine Weight as Percentage of Body Wt.
Alfalfa frozen, stored and then dried				
Veg. stage	20	5	10.32	.07381
Hay stage	20	5	9.92	.06815
Regrowth stage	20	5	11.24	.07497
Alfalfa dried immediately after harvest and stored				
Veg. stage	20	5	10.54	.06522
Hay stage	20	5	11.74	.07535
Regrowth stage	20	5	10.56	.07273
Sainfoin seed	20	5	11.76	.08885
Control	0	5	11.48	.08234

1/ Differences between treatments were not significant at P = .05.

activity from any of the alfalfa treatments. The mice wasted a large amount of the fibrous material in the rations which would tend to reduce the accuracy of the determination even if estrogenic activity had been observed.

No significant differences in uterine response between legumes or treatment levels occurred when six different levels of vegetative stages of alfalfa and red clover were tested using the Bickoff extraction procedure (6) as part of the mouse ration, Table XII. Bickoff et al. (6) recommends that the extract from 8 to 10 grams of forage be added to each 100 grams of ration. Levels of extract ranged from 5 grams per 100 grams of ration to 80 grams per 100 grams of ration without producing an indication of estrogenic activity. Red clover in the vegetative stage was later shown to have the highest estrogenic activity of all the stages of forages (Table XIV). The failure of the Bickoff extraction procedure (6) could have been due to many factors. Estrogenic compounds occur as minute quantities in forage even though their effect on animals is sometimes quite important. Estrogens are easily destroyed by heat and this could have occurred during extraction. The heat was kept as low as possible to produce evaporation of the acetone and alcohol, but may have been too high at times. Extracts from three separate extractions of red clover were used in trying to establish a dose level for the Bickoff extraction procedure (6) and none of them produced a response indicating the presence of estrogenic activity. At least one of these extracts should have had a small amount of estrogenic activity when compared to the results obtained from

Table XII. Estrogenic activity of increasing levels of extract from vegetation stages of alfalfa and red clover using Bickoff Fresh Forage Extraction Method.1/

Level of extract fed per 100 grs. of control ration	Actual grams of dry matter extract in feed	Number of mice per treatment	Average uterine wt. in milligrams	Average uterine wt. as percentage of body weight
Alfalfa				
5 grams	00.88	5	10.48	.07527
10 grams	1.75	5	11.12	.08184
15 grams	2.63	5	14.04	.10373
20 grams	3.50	5	11.52	.08372
40 grams	7.00	5	11.22	.07985
80 grams	14.00	4	11.70	.08526
Red Clover				
5 grams	00.75	5	16.30	.10249
10 grams	1.50	5	12.38	.09206
15 grams	2.25	5	10.84	.08534
20 grams	3.00	5	11.42	.07885
40 grams	6.00	5	12.80	.09306
80 grams	12.00	5	12.04	.08605
Control Ration	0	5	11.70	.09740

1/ Differences between crops, crops and control, or treatment levels were not significant at $P = .05$.

the Canadian extraction method (38) which is a much less refined technique. These results will be discussed later.

Two separate trials were run with three stages of alfalfa using the Canadian extraction procedure (38). Significant differences in estrogenic activity were produced both times showing the regrowth stage of alfalfa to be high in estrogenic activity (Table XIII). No significant differences occurred between the two methods of storage used on the hay stage.

The repeatability of a properly conducted bio-assay procedure is shown in Table XIII.

The next experiment containing eight legume forages at three stages of growth (vegetative, hay and regrowth) and sainfoin seed extracted by the Canadian method (38) also produced positive results (Table XIV). The three growth stages of red clover (vegetative, hay and regrowth) and alfalfa in the regrowth stage produced significant differences in estrogenic activity. Red clover in the vegetative stage was significantly more estrogenic than the other two stages of red clover or the alfalfa regrowth stage. Sainfoin forage extract in any of the three stages and sainfoin seed did not show a significant response. All the mice on the vegetative and regrowth stages of sickle milkvetch died while on experiment. This was due to an undetermined substance in the sickle milkvetch extract.

Significant differences resulted from the experiment containing the nine forages in the hay stage using the Bickoff extraction method (6) (Table XV). However, these were not reliable when compared to the same forage using the Canadian extraction procedure (38). Red clover shows

Table XIII. Estrogenic activity of three stages of alfalfa during two trials using the Canadian Extraction Method.1/

Treatment	Actual grams of dry matter extract in feed	Number of mice per treatment	Average uterine wt. in milligrams	Average uterine wt. as percentage of body weight
		Trial No. 1		
Alfalfa				
Veg. stage	60	5	8.74	.06455 b
Hay stage	60	5	10.14	.07969 b
Regrowth stage	60	5	19.46	.13977 a
Control	0	5	11.48	.08234 b
Hay stage (summer dried)	60	5	9.84	.07590 b
		Trial No. 2		
Alfalfa				
Veg. stage	60	5	11.48	.08834 b
Hay stage	60	5	10.94	.07643 b
Regrowth stage	60	5	15.54	.11341 a

1/ Values followed by same letter are not significant at P = .05.

Table XIV. Estrogenic activity of three stages of legumes and sainfoin seed using the Canadian Extraction Method.1/

Treatment	Date of Harvest	Stage of Growth	Dry Matter Percentage	Number of Mice Per Treatment	Average Uterine Wt. in Milligrams	Average Uterine Wt. as Percentage of Body Weight
Red clover	6-11-62	veg.	13.8	5	22.34	.20845 a
Alfalfa	6-11-62	veg.	17.8	5	11.48	.08834 b
Cicer mv.	6-11-62	veg.	15.5	5	7.98	.06594 b
Ladino clover	6-11-62	veg.	11.5	5	7.36	.06179 b
Alsike clover	6-11-62	veg.	12.0	5	7.74	.06057 b
Sainfoin	6-11-62	veg.	17.1	5	7.64	.05682 b
B. trefoil	6-11-62	veg.	14.1	5	6.20	.05000 b
Sickle mv.	6-11-62	veg.	20.3	Mice all died	-----	--------
Red clover	6-29-62	full bloom	30.8	5	18.20	.12257 a
Sainfoin seed	-------	----------	----	5	12.68	.08176 b
Alfalfa	6-27-62	5% bloom	33.3	5	10.94	.07643 b
Cicer mv.	7-5-62	50% bloom	24.4	5	11.46	.07253 b
Ladino clover	7-3-62	full bloom	23.6	5	10.86	.07032 b
B. trefoil	6-20-62	75% bloom	18.6	4	9.48	.06975 b
Sickle mv.	6-27-62	pod forming	27.2	5	7.56	.06697 b
Alsike clover	7-2-62	full bloom	22.1	5	9.58	.06657 b
Sainfoin	6-26-62	50% bloom	28.8	5	9.84	.06650 b
.0067 D.E.S.	control ration			5	17.50	.15735
Alfalfa	8-21-62	15% bloom	27.2	5	15.54	.11341 a
Red clover	8-21-62	50% bloom	22.3	5	13.84	.11273 a
Sainfoin	8-21-62	10% bloom	22.1	5	10.54	.07972 b
B. trefoil	8-21-62	90% pods	25.3	5	9.18	.06883 b
Alsike clover	8-21-62	10% bloom	20.7	5	9.22	.06875 b
Cicer mv.	8-21-62	veg.	19.2	5	9.10	.06533 b
Ladino clover	8-21-62	35% bloom	17.8	5	8.00	.05965 b
Sickle mv.	8-21-62	veg.	25.0	Mice all died	----	--------

1/ Values within a growth stage followed by the same letter are not significant at P = .05. Values underlined are significant to P = .05 within their particular legime.

Table XV. Estrogenic activity comparison of Bickoff Fresh Forage Extraction Method with Canadian Dry Forage Extraction Method on hay forage stage of various legumes.1/

Treatment	Actual grams of dry matter extraction feed	Number of mice per treatment	Average uterine wt. in milligrams	Average uterine wt. as percentage of body weight
Bickoff Extraction Method 27.75 grams of forage per 100 grams of control ration				
Alfalfa	5.25	5	10.76	.08772 a
Control	0	5	11.48	.08234 a b
Sainfoin	4.63	5	7.88	.07099 a b c
Ladino clover	4.10	5	9.06	.06908 a b c
B. trefoil	2.87	4	9.43	.06714 b c
Sickle mv.	4.42	5	9.18	.06487 b c
Orchardgrass	6.43	5	8.48	.06445 b c
Cicer mv.	3.88	5	7.98	.06273 c
Red clover	4.79	5	8.48	.06264 c
Alsike clover	3.54	5	7.50	.05670 c
Canadian Dry Extraction Method				
Red clover	60	5	18.20	.12257 a
Alfalfa	60	5	10.94	.07643 b
Cicer mv.	60	5	11.46	.07253 b
Ladino clover	60	5	10.86	.07032 b
B. trefoil	60	5	9.48	.06975 b
Sickle mv.	60	5	7.56	.06697 b
Alsike clover	60	5	9.58	.06657 b
Sainfoin	60	5	9.84	.06650 b

1/ Values followed by same letter are not significant at $P = .05$.

no estrogenic activity in the first procedure and exhibited significant activity in the second (Canadian) procedure. The author would recommend use of the Canadian method if further research is conducted with forage estrogens as it requires less precise chemical methods.

The estimated diethylstilbestrol equivalent potency of the estrogenic producing forages was calculated by regression (Figure 1). The regression line was calculated using the results of the first three levels of diethylstilbestrol on the dose response curve and the control ration. The diethylstilbestrol check used in the experiment with all stages of forages was compared to this line and then was used as part of the regression formula to calculate the estimated micrograms of diethylstilbestrol per pound of feed needed to produce the same response as produced by the forage (Table XVI). Red clover in the vegetative stage expressed a significantly higher equivalent in micrograms of diethylstilbestrol per pound of dry matter than any of the other forage stages which possessed estrogenic activity. These values are similar to those obtained in other research (21), (32), (38).

Sainfoin seed and forage in three growth stages did not show any significant estrogenic activity when compared with the other legumes.

Table XVI. Estimated diethylstilbestrol equivalency of red clover and alfalfa in micrograms per pound of dry matter.1/

Stage of growth and legume	Estimated potency in micrograms per pound of dry matter
Red clover veg.	6.78 a
Alfalfa regrowth	2.12 b
Red clover hay	1.87 b
Red clover regrowth	1.31 b

1/ Values followed by same letter are not significant at P = .05.

SUMMARY

Sainfoin is a deep-rooted, long-lived, tetrapoind perennial legume particularly adapted to dry, well-drained calcareous soils. It is reported not to cause bloat and outyielded all other legumes at the Montana Agricultural Experiment Station when harvested for only one cutting of hay.

An experiment was conducted comparing sainfoin and alfalfa seedling emergence from eight different seeding depths. Sainfoin was found to produce a significantly higher percentage of seedlings than alfalfa from a seeding depth of three inches or less. Seedling emergence of both species significantly decreased from a seeding depth of two inches or more. The variety by seeding depth interaction exceeded P - .05.

Three-week-old female white rats were used in a feeding trial to evaluate the protein quality of sainfoin seed. Sainfoin seed meal was found to be comparable in protein quality to soybean oil meal and thus warrants consideration as a source of supplemental protein for livestock feeding.

An experiment was carried out to compare the seasonal chemical composition of sainfoin with Ladak alfalfa, tetrapoid alsike clover, Tana birdsfoot trefoil, cicer milkvetch, Ladino clover, red clover, sickle milkvetch and orchardgrass. The data was collected from weekly samples of forage taken from plots at the Montana Agricultural Experiment Station, Crops and Soil Field Research Laboratory. The seasonal protein content of sainfoin was found to decrease with advancing maturity at about the same rate as the other crops. Dry matter percentage increased as protein percentage decreased. A high negative correlation (-.89) was calculated for the

relation of protein to dry matter percentage. This could lead to a quick, comparative method of estimating crude protein content of legume forages. Sainfoin matures at about the same rate as alfalfa. It has a higher leaf to stem ratio than alfalfa and is lower in crude fiber and protein. Sainfoin has a high percentage of nitrogen free extract in comparison to alfalfa which, coupled with its lower protein content and high yield, makes it worthy of consideration as a silage crop.

A bio-assay for estrogenic activity using immature female white mice was conducted with three growth stages (vegetative, hay, and regrowth) of the same legumes used for comparisons of seasonal chemical composition. The forage samples were collected and stored in a frozen state until extraction. Two methods of extraction were used. The extracts were added to a control ration and fed to the mice for seven days. The Canadian method of Extraction proved to be more reliable than the Bickoff method for extracting fresh forage. All three stages of red clover and the regrowth stage of alfalfa produced a significantly higher estrogenic response than any of the other legumes. Sainfoin did not exhibit a measurable amount of estrogenic activity in any stage of growth.

Sainfoin possesses potential for both forage and seed production in Montana, especially in dryland areas where one cutting of hay is harvested or on irrigated land where a high-yielding silage crop is desired.

LITERATURE CITED

1. Alfonsus, L. 1949. Sainfoin next to the clovers as a honey plant. Am. Bee Jour. 69:113-114.

2. Anderson, J. O. 1962. Amino acids in nutrition. Utah Farm and Home Sci. 23:38-39.

3. Anonymous. 1923. Sainfoin. Jour. Ministry of Agr. 30:426:430.

4. Baker, C. J. L. 1952. Studies on the composition of sainfoin. Jour. Agr. Sci. 42:382-394.

5. Beveridge, J. L. and B. P. Wilsie. 1959. Influence of depth of planting, seed size, and variety on emergence and seedling vigor in alfalfa. Agron. Jour. 51:731-734.

6. Bickoff, E. M., A. N. Booth, R. L. Lyman, A. L. Livingston and A. P. Hendrickson. 1959. Determination of estrogenic activity in fresh and dried forages. Jour. An. Sci. 18:1000:1009.

7. Bickoff, E. M., A. N. Booth, A. L. Livingston, A. P. Hendrickson and G. O. Kohler. 1960. Estrogenic activity in dehydrated and suncured forages. Jour. An. Sci. 19:189-197.

8. Bickoff, E. M., A. N. Booth, A. L. Livingston and A. P. Hendrickson. 1960. Observations on the effect of drying on estrogenic activity of alfalfa samples of varying maturity. Jour. An. Sci. 19:745-753.

9. Bickoff, E. M. 1961. Estrogen like substances in plants. Western Regional Research Laboratory, Albany 10, California. Excerpts from speech.

10. Cooper, C. S. 1961, 1962. Annual Reports - Forage Yields. United States Department of Agriculture Agronomist, Montana State College, Bozeman, Montana.

11. Dadant, C. P. 1928. Sainfoin for bees. Am. Bee Jour. 68:180.

12. Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics 11:1-42.

13. Erickson, L. C. 1946. The effect of alfalfa seed size and depth of planting upon the subsequent procurement of stand. Jour. Am. Soc. Agron. 38:964-73.

14. Eslick, R. F. 1951. Fiftieth - - - Fifty Ninth Annual Reports of the Montana Agricultural Experiment Station. July 1, 1942 - June 30, 1950. P. 12.

15. Eslick, R. F. 1962 Unpublished data on sainfoin. Montana State College Plant and Soil Science Department.

16. Evans, A. M. 1961. The yields of eight varieties of sainfoin cut at different growth stages. Emp. Jour. Exp. Agr. 49:323-329.

17. Fyfe, J. L. 1946. Polyploidy in sainfoin. Nature. 158:418.

18. Fyfe, J. L. 1943. Sainfoin (a very useful crop). Jour. Ministry of Agr. 49:208-10.

19. Kamstra, L. D. 1961. Taking plants apart to understand their value as a feed. S. D. Agr. Exp. Farm and Home Research. 12:26-28.

20. Kitts, W. D., E. Swierstra, V. C. Brink and A. J. Wood. 1959. The estrogen like substances in certain legumes and grasses. I. The quantitative determination of such substances in red clover and oats. Canadian Jour. An. Sci. 39:6-13.

21. Kitts, W. D., E. Swierstra, V. C. Brink and A. J. Wood. 1959. The estrogen like substances in certain legumes and grasses. II. The effect of stage of maturity and frequency of cutting on the estrogenic activity of some forages. Canadian Jour. An. Sci. 39:158-163.

22. Kumpta, J. S. and A. E. Harper. 1961. Amino acid balance and imbalance. Jour of Nut. 74:2, p. 139-146.

23. Legg, S. P, D. H. Curnow and S. A. Simpson. 1950. The seasonal and species distribution of oestrogen in British pasture plants. Bio-chem. Jour.

24. Madoc, E. W. D. 1934. Sainfoin, fodder and honey plant. Am. Bee Jour. 74:201.

25. Manley, R. B. 1926. Facts about sainfoin. Am. Bee Jour. 66:378.

26. Mansfield, W. S. 1945. Lucerne and sainfoin. Jour. Ministry of Agr. 52:255-258.

27. Morrison, F. B. 1956. Feeds and Feeding, 22nd Edition. Morrison Publishing Co. Ithaca, New York.

28. Morse, W. J. 1950. Soybean Production for Hay and Beans. U.S.D.A. Farmers Bulletin 2024.

29. Norton, J. E. 1931. Irrigated Alfalfa in Montana. Bulletin 245. Montana Agricultural Experiment Station.

30. Owen, E. B. 1956. The storage of seeds for maintainance of viability. Bulletin 43, Commonwealth Agricultural Bureau. pp. 15 and 44.

31. Pellet, F. C. 1940. Sainfoin - the hay and honey plant. Am. Bee Jour. 80:362-363.

32. Pieterse, P. J. S. and F. N. Andrews. 1956. The estrogenic activity of alfalfa and other feedstuffs. Jour. An. Sci. 15:25-36.

33. Pieters, A. T. '1937. Effect of maturity on chemical composition of leguminous forage plants. Jour. Am. Soc. Agron. 29:436-440.

34. Piper, C. V. 1927. Forage Plants and Their Culture. Chap. 23, pp. 492-495. Macmillan Company, New York.

35. Rama Rao, P. B., V. Metts, H. W. Norton, and B. C. Honson. 1958. Minimum essential amino acid requirements of rats. Am. Jour. of Clin. Nut. 6:487-489.

36. Reid, J. T. 1960. Effects of growth stage, chemical composition and physical properties upon the nutritive value of forages. Jour. Dairy Sci. 42:567-571.

37. Rules for Testing Seeds. 1960. Association of Official Seed Analysis. 49:2-4.

38. Swierstra, E. 1958. The estrogen-like substances in various legumes and grasses, and the effect of these compounds on the reproduction and growth of certain laboratory animals. Master's thesis. University of British Columbia.

39. Thomson, J. R. 1952. Further seed studies in sainfoin. Brit. Grassland Soc. Jour. 7:65-69.

40. Thomson, J. R. 1951. Seed studies in sainfoin. Brit. Grassland Soc. Jour. 6:147-159.

41. Triplett, G.B., Jr. and M. B. Tesar. 1960. Effects of compaction, depth of planting, and soil moisture tension on seedling emergence of alfalfa. Agron. Jour. 52:681-684.

42. Woodman, H. E. and R. E. Evans. 1947. The chemical composition and nutritive value of rye grass seed meal, clover seed meal, lucerne seed meal and sainfoin seed meal. Jour. Agr. Sci. 37:311-315.

43. Young, L. G. and T. Dunn. 1962. Unpublished data on rats fed pigweed seed. Montana State College, Animal Science Department.

44. Youngman, V. E. 1962. Investigations on the estrogenic activity of selected forage crops. Doctor's thesis, Washington State University.