The Use of Sesquiterpene Lactones as Taxonomic Markers in the Shrubby Species of *Artemisia* (Section *Tridentatae*) in Montana

RICK G. KELSEY, MELVIN S. MORRIS, AND FRED SHAFIZADEH

Highlight: *The aerial parts of sagebrush contain many interesting natural products. The sesquiterpene lactones are a class of compounds that can be easily extracted from these plants and then analyzed by thin-layer chromatography. When used in combination with the morphological characteristics, the sesquiterpene lactones can provide useful taxonomic markers that aid in separating various sagebrush taxa.*

In 1965, Young reported the results of his chemical survey of 16 sagebrush taxa from Sublette County, Wyoming. This work led the way to the discovery of a new subspecies within the *Artemisia tridentata* complex, the subspecies *wyomingensis* (Beetle and Young, 1965). Since then, there has been a tremendous interest in the chemical makeup of the woody species of sagebrush. In particular, the chemical constituents have been investigated in search of additional characteristics that might be useful in the identification or the classification of these species.

Researchers in other states have conducted surveys similar to Young's. Holbo and Mozingo (1965) and Brunner (1972) examined many species in Nevada, while Winward (1970) studied the *A. tridentata* complex in Idaho. These studies were very similar in that they all dealt with the ultraviolet fluorescent compounds, now known to be coumarins and flavonoids (Shafizadeh and Melnikoff, 1970; Rodriquez et al., 1972; and Brown et al., 1975), and they all to some degree found these compounds to be useful taxonomic markers. In addition to being useful taxonomic markers, the ultraviolet fluorescent chemicals have been shown to be reliable indicators of palatability differences in sagebrush (Hanks et al., 1971, 1973; Hanks and Jorgensen, 1973; Stevens and McArthur, 1974). Hanks et al. (1973) have also used these compounds as a basis for inferring phylogenetic relationships.

For the last several years, a previously uninvestigated class of compounds, the sesquiterpene lactones, have been the subject of some intense studies in sagebrush (Irwin, 1971; Shafizadeh et al., 1971; Kelsey et al., 1973; Kelsey, 1974). The sesquiterpene lactones have been used to help solve both taxonomic and phylogenetic problems in other genera, particularly members of the *Compositae* (Payne et al., 1973; Yoshioka et al., 1973). In Montana, we have directed our studies toward determining the systematic usefulness of the sesquiterpene lactones in the sagebrush species described by Beetle (1960). Although the coumarins and flavonoids have been useful taxonomic markers, they have not provided answers for all the systematic questions associated with the woody *Artemisias*. The sesquiterpene lactones may provide new and independent characteristics that may be used with morphology, ecology, cytology, and the flavonoids and coumarins for solving some of the systematic problems. In this report, we discuss how the sesquiterpene lactones have been useful as taxonomic markers in Montana sagebrush species.

Materials and Methods

To extract the sesquiterpene lactones, cover 10 grams of crushed air-dried leaves with 100 ml of chloroform and let stand for approximately 48 hours; periodic shaking or stirring will enhance the extraction. After two days, filter off the leaves and evaporate the chloroform; this yields a thick green residue. For the best analytical results, it is recommended that this residue be further processed as outlined in Figure 1. However, it is possible to detect many of the sesquiterpene lactones by thin-layer chromatography (TLC) without further purification of the initial chloroform extract.

The sesquiterpene lactones are analyzed by TLC on 20 × 20 cm silica gel plates covered with 0.50 mm of silica gel G (Woelm).[1] Commercially prepared plates can also be used. Prior to use all plates are heated for 30 minutes in a 100–110°C oven. Just before applying the sesquiterpene lactones to the TLC plate, add a few drops of chloroform to the extract so that it can be drawn up into a small capillary tube. Two or three drops of the solution should be sufficient for analysis. The samples can then be spotted 2.0 cm from the base of the plate and approximately 1.0 cm apart; this will allow 18–20 samples to be run simultaneously.

After all the samples have been applied, place the plate in the solvent chamber. Our major solvent system is chloroform:petroleum ether:ethyl acetate (2:2:1, V/V/V); however, this separates only the nonpolar compounds. More polar compounds can be detected by using

The authors are, respectively, research associate with the Wood Chemistry Laboratory, professor emeritus of range management, School of Forestry, and director of the Wood Chemistry Laboratory, University of Montana, Missoula, 59801.

Manuscript received October 25, 1975.

[1]Manufactured by M. Woelm, Eschwege, Germany. Exclusively distributed by ICN Pharmaceuticals, Inc., Cleveland, Ohio. Other brands of silica gel G are satisfactory.

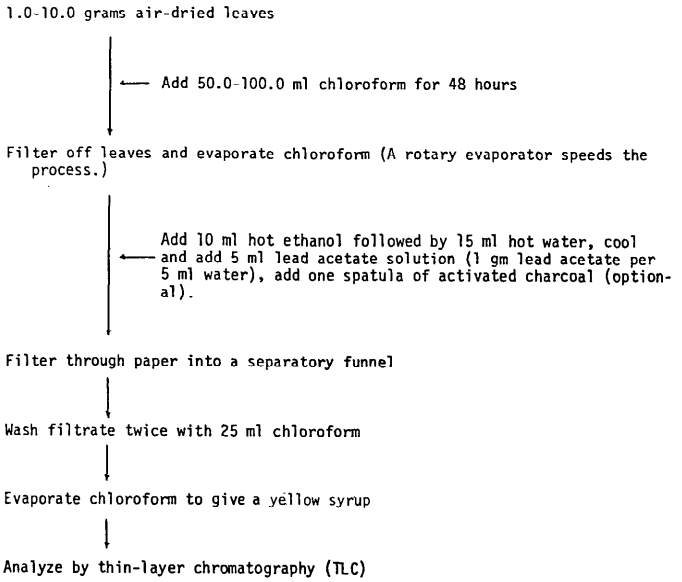
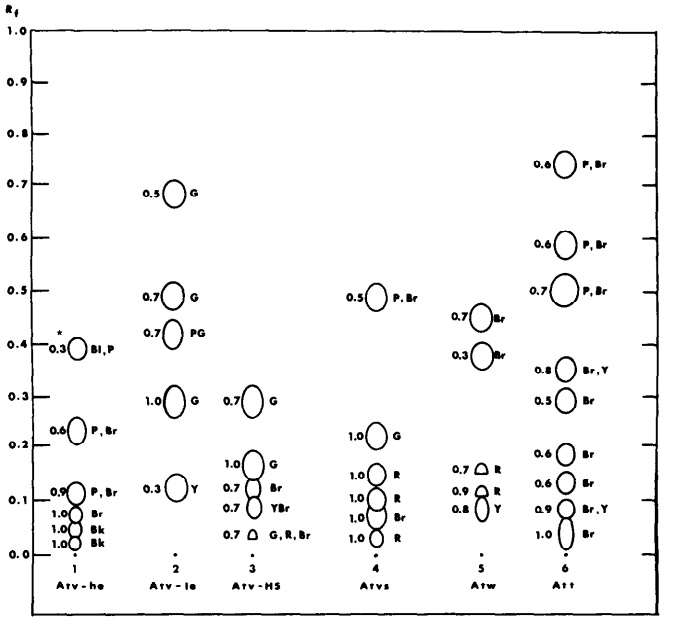


Fig. 1. *The procedure for extracting sesquiterpene lactones from sagebrush.*



Pattern No.	*Species*
1	*A. tridentata* ssp. *vaseyana* high elevation
2	*A. tridentata* ssp. *vaseyana* low elevation
3	*A. tridentata* ssp. *vaseyana* Hot Springs
4	*A. tridentata* ssp. *vaseyana* f. *spiciformis*
5	*A. tridentata* ssp. *wyomingensis*
6	*A. tridentata* ssp. *tridentata*

*The number at the left of each spot is the frequency of that spot. Frequency = the no. of samples of one species in which the spot was recorded in greater than trace amounts/total no. of samples of that species or chemical type examined.

Color Code—Bl-blue, P-Purple, Br-Brown, Bk-Black, G-Green, PG-Purple Green, Y-Yellow, YBR-Yellow Brown, R-Red, OBR-Orange Brown, O-Orange, RP-Reddish Purple. A comma between two colors, i.e., P,Br means the spot may be purple or brown in color.

Fig. 2. *The diagnostic sesquiterpene lactone TLC patterns for the subspecies of* A. tridentata.

chloroform:petroleum ether:ethanol (5:4:1). The results of this paper are based entirely on the first solvent system. The plate should remain in the chamber until the solvent front has moved approximately 15.0 cm beyond the point of sample application. Mark the solvent front; remove the plate from the chamber and allow it to air-dry. Sesquiterpene lactones are not detectable in visible light. For compounds to be visible, the surface of the plate must be sprayed with a fine mist of concentrated sulfuric acid and heated in an oven set at 100–110°C for 3–5 minutes. The sesquiterpene lactones can exhibit a variety of colors (green, blue, red, yellow, brown, purple, etc.) and the length of the heating period required to make each compound visible may vary. Some sesquiterpene lactones will become visible immediately upon spraying with sulfuric acid. Others show up after 1–5 minutes of heating, and there are a few that require extensive heating in excess of 5 minutes. Long heating periods, however, will result in the charring and loss of color for most compounds. The best way to prevent overlooking compounds due to insufficient heating is to place the plate back into the oven after recording.

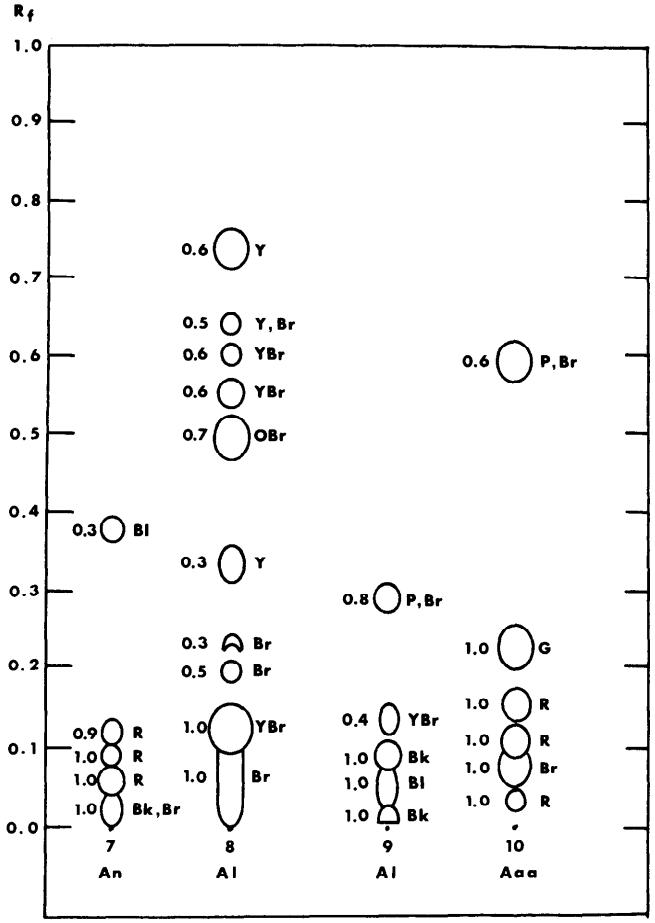
Plates should be removed from the oven when the colors are very bright, as stated above, usually with in 1–5 minutes, depending on the samples. The colors produced by the sesquiterpene lactones are stable for an hour or so; thereafter, they begin to fade slowly. The intense colors observed immediately after heating can be maintained for longer periods by covering the surface of the chromatogram with a glass plate. The Rf values (distance moved by compound/distance moved by solvent front) and the colors of each spot should be recorded soon after removing the chromatogram from the oven. We have noted that colors develop more rapidly on commercially prepared plates, so they must be watched very closely while heating at 100–110°C, or the temperatures should be turned down.

Results and Discussion for Montana

The diagnostic TLC patterns for the species and subspecies of sagebrush common in Montana are illustrated in Figures 2, 3, and 4. It is important to realize that only the most diagnostic spots, those with a frequency of 29% and greater (frequency = number of samples of one species in which the spot was recorded in greater than trace amounts/total number of samples of that species or chemical type examined), have been diagramed. Most chromatograms will have more spots, particularly at the higher Rf values. However, these are usually extremely variable and of little use. Frequently, a single compound on the chromatogram will exhibit slightly different shades of color, such as light purple or brown; consequently, the large number of spots marked as both purple or brown. However in some taxa, more than one compound, each with a different color, occur at approximately the same Rf, resulting in a variety of colors being recorded. This is illustrated by the lowest spot in *A. tridentata* ssp. *vaseyana* Hot Springs (pattern 3, Fig. 2).

Sesquiterpene lactones can be extracted from sagebrush plants at any time of the year, although samples collected during the summer months, particularly August and September, provide the best results. Our studies have shown that the young branches, influorescence, and seeds contain the same sesquiterpene lactones as the leaves, although leaves are the easiest to handle. The biological production of the sesquiterpene lactones in sagebrush appear to be genetically controlled and not random or haphazard (Kelsey, 1974). Their consistency and reproducibility have demonstrated that these compounds are significant characteristics for use in sagebrush systematics. The fact that the sesquiterpene lactones vary quantitatively does not refute genetic control or eliminate their systematic usefulness because quantity like quality may also be genetically controlled.

With few exceptions, in order for the chromatography of the sesquiterpene lactones to be used as an effective taxonomic tool, it must be used in conjunction with the morphological characteristics. The best technique is to identify the specimens in the field using as many morphological and ecological criteria as possible and then using the chemical analysis as an additional check or



Pattern No.	*Species*
7	*A. nova*
8,9	*A. longiloba*
10	*A. arbuscula* ssp. *arbuscula*

Color Code—See Fig. 2.

Fig. 3. *The diagnostic sesquiterpene lactone TLC patterns for* A. nova, A. longiloba, *and* A. arbuscula.

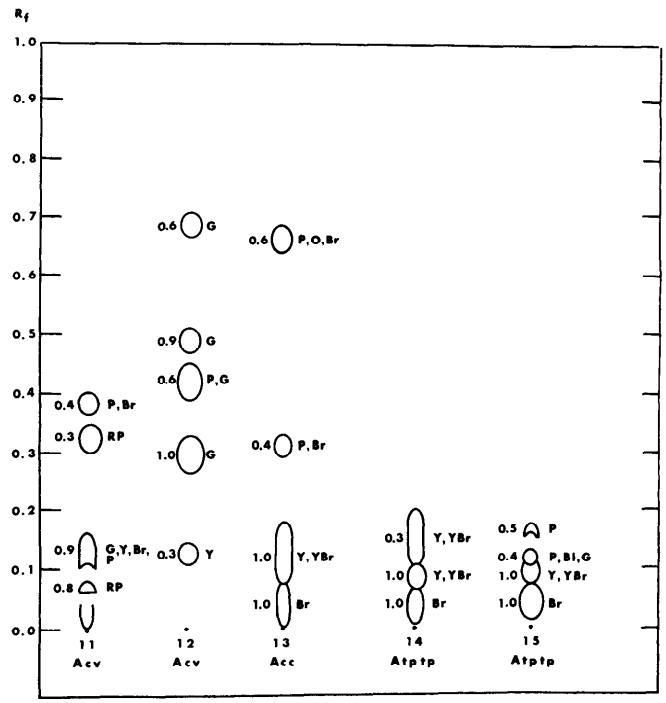
confirming characteristic. Because of the similarity of chromatographic patterns between some taxa, the sesquiterpene lactones cannot separate all species of Montana sagebrush. However, if the species are separated initially by morphology into one of the following groups: (1) *A. tridentata*, (2) *A. cana*, (3) *A. tripartita*, or (4) others (containing *A. nova, A. longiloba,* and *A. arbuscula*), the sesquiterpene lactones can separate the species and subspecies within each category.

The sesquiterpene lactones have been extremely beneficial in separating the three subspecies within the big sagebrush (*Artemisia tridentata*) complex (Fig. 2). *Artemisia tridentata* ssp. *vaseyana* has been found to maintain three distinct sesquiterpene lactone races in western Montana (Shafizadeh et al., 1971; Kelsey et al., 1973). Two of these chemical races exhibited interesting topographic distributions and are probably ecotypes; they deserve further consideration ecologically. The high elevation sesquiterpene lactone race of the ssp. *vaseyana* (pattern 1, Fig. 2) is found predominately above 6,000 feet, while the low elevation race (pattern 2, Fig. 2) usually occurs below 6,400 feet elevation. There is a transition zone at 6,000–6,400 feet where both chemical races can be found growing intermixed. The third sesquiterpene lactone race of the ssp. *vaseyana* is referred to as the Hot Springs race (pattern 3, Fig. 2) because its geographic distribution is restricted to the isolated Hot Springs Valley in northwestern Montana. *Artemisia tridentata* ssp. *vaseyana* f. *spiciformis* (pattern 4, Fig. 2) produces a distinct set of sesquiterpene lactones which make it easy to separate from the above three chemical races in the ssp. *vaseyana*.

Artemisia tridentata ssp. *wyomingensis* has two TLC patterns, although 90% of the plants examined possessed pattern 5 in Figure 2, which could be easily distinguished from all the other TLC patterns in this species. The second pattern in the ssp. *wyomingensis* was not diagramed because of its low frequency of occurrence. *Artemisia tridentata* ssp. *tridentata* had one TLC pattern (pattern 6, Fig. 2) that could be used to separate it from the other two subspecies.

We would like to stress the importance of distinguishing big sagebrush at the subspecific level. It is apparent from our observations, as well as others (Winward, 1970; McDonough and Harniss, 1974, 1975) that the three subspecies, *vaseyana, tridentata,* and *wyomingensis,* differ enough ecologically to warrant subspecific identification.

In 1970, Beetle emphasized the distinctness among the three species, *A. longiloba, A. arbuscula,* and *A. nova.* The sesquiterpene lactones add support to the individuality of these three species. *Artemisia nova* had one diagnostic TLC pattern not found in any other species (pattern 7, Fig. 3). *Artemisia longiloba* had two patterns, one of which (pattern 8, Fig. 3), was difficult to separate from other species (*A. tridentata* ssp. *tridentata,* pattern 6, Fig. 2; *A. cana* ssp. *cana,* pattern 13, Fig. 4; *A. tripartita* ssp. *tripartita,* patterns, 14, 15, Fig. 4). The second one, however, was species specific (pattern 9, Fig. 3) (Shafizadeh and Bhadane, 1973a). *Artemisia arbuscula* ssp. *arbuscula* had one pattern (pattern 10, Fig. 3) that was easily differentiated from those in *A. longiloba* and *A. nova,* but it was identical to the pattern in *A. tridentata* ssp. *vaseyana* f. *spiciformis* (pattern 4, Fig. 2) (Shafizadeh and Bhadane, 1972a,



Pattern No.	*Species*
11,12	*A. cana* ssp. *viscidula*
13	*A. cana* ssp. *cana*
14,15	*A. tripartita* ssp. *tripartita*

Color Code—See Fig. 2.

Fig. 4. *The diagnostic sesquiterpene lactone TLC patterns for* A. cana *and* A. tripartita.

1973b; Kelsey et al., 1973). The brown spots, at Rf 0.60 in the *A. arbuscula* ssp. *arbuscula* pattern, and Rf 0.50 in *A. tridentata* ssp. *vaseyana* f. *spiciformis* pattern, were not diagnostic enough to separate these two taxa chemically. They are, however, quite easily separated by morphology.

Artemisia cana ssp. *viscidula* had two TLC patterns, one (pattern 11, Fig. 4) was species and subspecies specific (Shafizadeh and Bhadane, 1972b) and the other (pattern 12, Fig. 4) was identical to that of *A. tridentata* ssp. *vaseyana* low elevation sesquiterpene lactone race (pattern 2, Fig. 2). *Artemisia cana* ssp. *cana* (Bhadane and Shafizadeh, 1975) had one pattern (pattern 13, Fig. 4) that was subspecies specific separating the ssp. *cana* from the ssp. *viscidula*, but it was very difficult to distinguish the ssp. *cana* pattern from those in *A. tridentata* ssp. *tridentata* (pattern 6, Fig. 2), *A. tripartita* ssp. *tripartita* (patterns 14 and 15, Fig. 4), and one of the patterns from *A. longiloba* (pattern 8, Fig. 3).

Artemisia tripartita ssp. *tripartita* had two very similar patterns (patterns 14 and 15, Fig. 4) (Shafizadeh et al., 1974), that were nearly identical to the one in *A. cana* ssp. *cana*. Like *A. cana*, they were difficult to differentiate from *A. tridentata* ssp. *tridentata* and the one *A. longiloba* pattern discussed above.

It is apparent from these results that the thin-layer chromatography of the sesquiterpene lactones canot be used as the only criteria for sagebrush identification. The sesquiterpene lactones are very useful in separating species and subspecies that are easily confused on the basis of morphology, i.e., the subspecies within *A. tridentata*, and less useful in separating the morphological distinct taxa, i.e., *A. tripartita* ssp. *tripartita* and *A. cana* ssp. *cana*. Currently, the problem of chemical similarity between distinct taxa, such as *A. arbuscula* ssp. *arbuscula* and *A. tridentata* ssp. *vaseyana* f. *spiciformis*, remains unexplained. Hybridization, introgression, and convergent evolution might account for these phenomena.

We think that the sesquiterpene lactones are relatively easy to analyze and interpret for several reasons. First, they can be analyzed by one dimensional chromatography in rapidly moving solvent systems. Second, many of the sesquiterpene lactones exhibit a variety of colors, such as red, green, blue, etc. when sprayed with acid and heated. These color differences reflect structural differences in the compounds. Third, after spraying a TLC plate, the length of the heating period required to produce the visible colors can be diagnostic. These three characteristics make the chromatography of the sesquiterpene lactones a very attractive technique for obtaining chemical markers in shrubby *Artemisias*.

Conclusion

Our work has demonstrated that the sesquiterpene lactones can provide very useful taxonomic characteristics to supplement the morphology and ecology for identifying and separating sagebrush taxa within the state of Montana. However, except for the work of Irwin (1971) and Asplund et al. (1972), very little is known about the sesquiterpene lactones in sagebrush species from other areas. Any general conclusions regarding the systematic usefulness of these compounds must await a larger work on sagebrush species over their entire ranges.

Literature Cited

Asplund, R. O., M. McKee, and P. Balasubramaniyan. 1972. Artevasin: a new sesquiterpene lactone from *Artemisia tridentata*. Phytochemistry 11: 3542:3544.

Beetle, A. A. 1960. A study of sagebrush: *Tridentatae* of *Artemisia*. Wyo. Agr. Exp. Sta. Bull. 368. 83 p.

Beetle, A. A. 1970. An ecological contribution to the taxonomy of *Artemisia*. Mandroño 20:385-386.

Beetle, A. A., and A. Young. 1965. A third subspecies in the *Artemisia tridentata* complex. Rhodora 67:405-406.

Bhadane, N. R., and F. Shafizadeh. 1975. Sesquiterpene lactones of sagebrush: the structure of artecanin. Phytochemistry 14:2651-2653.

Brown, D., R. O. Asplund, and V. A. McMahon. 1975. Phenolic constituents of *Artemisia tridentata* ssp. *vaseyana*. Phytochemistry 14: 1083-1084.

Brunner, J. R. 1972. Observations on *Artemisia* in Nevada. J. Range Manage. 25:205-208.

Hanks, D. L., J. R. Brunner, D. R. Christensen, and A. P. Plummer. 1971. Paper chromatography for determining palatability differences in various strains of big sagebrush. U.S. Dep. Agr., Forest Serv., Intermountain Forest and Range Exp. Sta. Res. Pap. INT-101. 9 p.

Hanks, D. L., and K. R. Jorgensen. 1973. Chromatographic identification of big sagebrush seed. J. Range Manage. 26:304.

Hanks, D. L., E. D. McArthur, R. Stevens, and A. P. Plummer. 1973. Chromatographic characteristics and phylogenetic relationships of *Artemisia*, section *Tridentatae*. U.S. Dep. Agr., Forest Serv., Intermountain Forest and Range Exp. Sta. Res. Pap. INT-141. 24 p.

Holbo, H. R., and H. N. Mozingo. 1965. The chromatographic characterization of *Artemisia*, section *Tridentatae*. J. Bot. 52:970-978.

Irwin, M. A. 1971. Sesquiterpene lactones of *Artemisia*. PhD Diss. Univ. of California, Los Angeles.

Kelsey, R. G. 1974. The systematic usefulness of the sesquiterpene lactones in the genus *Artemisia*, section *Tridentatae*, (sagebrush) of Montana. PhD Diss. Univ. of Montana, Missoula.

Kelsey, R. G., M. S. Morris, N. R. Bhadane, and F. Shafizadeh. 1973. Sesquiterpene lactones of *Artemisia*: TLC analysis and taxonomic significance. Phytochemistry 12:1345-1350.

McDonough, W. T., and R. O. Harniss. 1974. Effects of temperature on germination in three subspecies of big sagebrush. J. Range Manage. 27:204-205.

McDonough, W. T., and R. O. Harniss. 1975. Know your sagebrush—and better your range. Utah Sci. 36:99-103.

Payne, W. W., T. A. Geissman, A. J. Lucas, and T. Saitoh. 1973. Chemosystematics and taxonomy of *Ambrosia chamissonis*. Biochemical Systematics 1:21-33.

Rodriquez, E., N. J. Carman, G. Vander Velde, J. H. McReynolds, T. J. Mabry, M. A. Irwin, and T. A. Geissman. 1972. Methoxylated flavonoids from *Artemisia*. Phytochemistry 11: 3509-3514.

Shafizadeh, F., and N. R. Bhadane. 1972a. Badgerin, a new germacranolide from *Artemisia arbuscula* ssp. *arbuscula*. J. Org. Chem. 37:274-277.

Shafizadeh, F., and N. R. Bhadane. 1972b. Sesquiterpene lactones of sagebrush. New guaianolides from *Artemisia cana* ssp. *viscidula*. J. Org. Chem. 37:3168-3173.

Shafizadeh, F., and N. R. Bhadane. 1973a. Longilobol, a new sesquiterpene triol from *Artemisia longiloba* (Osterhout) Beetle. Tetrahedron Letters No. 24. p. 2171-2174.

Shafizadeh, F., and N. R. Bhadane. 1973b. Sesquiterpene lactones of *Artemisia arbuscula* and *A. tridentata*. Phytochemistry 12:857-862.

Shifizadeh, F., N. R. Bhadane, and R. G. Kelsey. 1974. Sesquiterpene lactones of sagebursh: constituents of *Artemisia tripartita*. Phytochemistry 13:669-670.

Shafizadeh, F., N. R. Bhadane, M. S. Morris, R. G. Kelsey, and S. N. Khanna. 1971. Sesquiterpene lactones of big sagebrush. Phytochemistry 10:2745-2754.

Shafizadeh, F., and A. B. Melnikoff. 1970. Coumarins of *Artemisia tridentata* ssp. *vaseyama*. Phytochemistry 9:1311-1316.

Stevens, R., and E. D. McArthur. 1974. A simple field technique for identification of some sagebrush taxa. J. Range Manage. 27:325-326.

Winward, A. H. 1970. Taxonomic and ecological relationships of the big sagebrush complex in Idaho. PhD Diss. Univ. of Idaho, Moscow.

Yoshioka, H., T. J. Mabry, and B. N. Timmermann. 1973. Sesquiterpene lactones. Univ. of Tokyo Press. p. 85-114.

Young, A. L. 1965. A chemical study of the taxonomy of the section *Tridentatae* of the genus *Artemisia*. Wyoming Range Manage. Issue 198:2-9.