Phytochemistry, Vol. 24, No. 10, pp. 2233–2237, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

INFRASPECIFIC VARIATION OF FOLIAGE DITERPENES OF *DACRYDIUM CUPRESSINUM*

NIGEL B. PERRY and REX T. WEAVERS

Department of Chemistry, University of Otago, Box 56, Dunedin, New Zealand

(*Revised received* 20 *March* 1985)

Key Word Index—*Dacrydium cupressinum*; Podocarpaceae; infraspecific variation; diterpenes; lauren-1-ene; rimuene; phyllocladene; juvenile foliage; geographic variation.

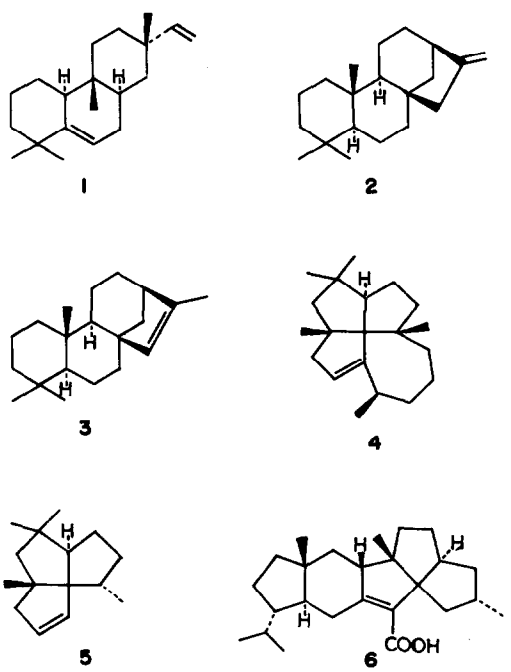
Abstract—The unique diterpene lauren-1-ene has only been found in the foliage of *Dacrydium cupressinum*, the New Zealand rimu, but the levels of this and other diterpenes vary greatly from tree to tree. Diterpene levels are apparently under genetic control, as they are not affected by environmental factors. Male and female trees do not differ significantly, but juvenile trees have lower phyllocladene levels than do adult trees. Different regions of New Zealand contain different proportions of high-laurenene trees.

INTRODUCTION

New Zealand has long been isolated from other land masses and this has led to the development of very distinctive flora and fauna [1]. About 80% of New Zealand's plant species do not occur anywhere else in the world [2] and all twenty native conifer species, from the families Araucariaceae, Cupressaceae and Podocarpaceae, are endemic [3, 4]. Two of these families, the Araucariaceae and the Podocarpaceae are largely restricted to the southern hemisphere and are thought to have evolved separately from northern families such as the Pinaceae [5]. New Zealand's conifers have proved to be rich sources of diterpene hydrocarbons and oxygenated diterpenes [6, 7].

The most widely occurring forest tree in New Zealand is the rimu, *Dacrydium cupressinum* Sol. *ex* Lamb. [8]. A steam distillate of its foliage was the original source of (+)-rimuene (**1**) [9–11], and (+)-phyllocladene (**2**) and isophyllocladene (**3**) have also been reported [6, 12]. Foliage extracts show some insecticidal activity, but no growth inhibition of other plants [13, 14].

More recently, a highly unusual diterpene, (−)-lauren-1-ene (**4**) (called laurenene hereafter), was isolated from rimu foliage [15, 16]. This compound has not been reported from any other source and its structure, with a rosette arrangement of four carbocyclic rings, is unique among natural products. A similar arrangement was first achieved synthetically in 1972 and the term 'fenestrane' was proposed for this type of structure [17] (laurenene can be regarded as a [5.5.5.7]fenestrane). Laurenene bears little structural resemblance to any other diterpene; the natural products which it resembles most closely are the 'triquinane' [18] sesquiterpenes such as silphinene (**5**) [19] and the sesterterpene, retigeranic acid (**6**) [20]. When more laurenene was required for investigations of its unusual chemistry [21–25], several rimu trees had to be examined before another with a useful level of this compound was found. The variability in terpene composition which became apparent led directly to the present study.



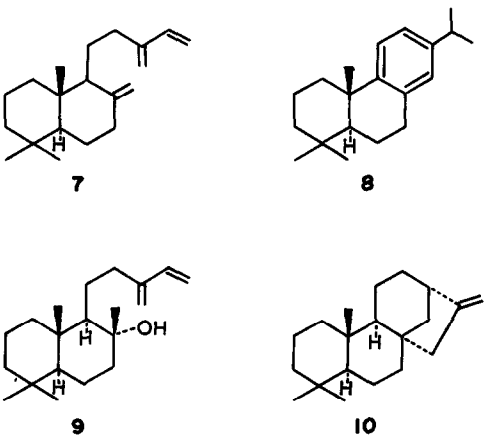
It is not unusual for individuals or populations within a species to have significantly different levels of secondary compounds [26, 27], and there have been several reported cases of infraspecific variation of diterpenes in the foliage of gymnosperms. A survey of the diterpene hydrocarbons of the foliage of the New Zealand Podocarpaceae found some examples of infraspecific variation [6] and similar phenomena have also been encountered in the Cupressaceae [28] and in the Taxodiaceae [29].

Davis and Heywood have discussed infraspecific variation in general terms [30] and they distinguish two types: variation between individuals within a population and variation between populations. The causes of variation

can be either environmental or genetic, and the following approach to a study of the variation in the levels of laurenene and the other diterpenes in *D. cupressinum* was decided upon. Areas for examination were:

1. The variation with position on a single tree, both in foliage from different positions, and in different organs. 2. Variations within a single population, checking for correlation with environmental factors. 3. Seasonal variation. 4. As *D. cupressinum* is dioecious, sex-related differences could occur. 5. Differences between the morphologically distinct juvenile and adult foliage. 6. Regional variation.

No hybridization of *D. cupressinum* with other species has been found [8] so this cause of genetic variation was ruled out. No interactions with other organisms (e.g. fungal infections), a possible environmental factor, were noticed in this study.



RESULTS

Gas chromatography using capillary columns, at present the best method for quantitative analysis of diterpene hydrocarbon mixtures [31], was used in this study. Diterpene levels are reported either by expressing the areas of the corresponding GC peak as percentages of the total diterpene peak areas, or relative to the area of an internal standard peak. Area percentages would correspond to mass percentages only if the relative molar responses (for definition, see von Rudloff [32] or Novak [33]) for each component were the same. This was not the case for laurenene, rimuene and phyllocladene (see Experimental).

The wide variation of laurenene (**4**) levels in the foliage of *D. cupressinum* is illustrated in Fig. 1. Rimu trees are clearly separated into three categories with high, medium and low laurenene levels. Rimuene (**1**) and phyllocladene (**2**) levels also varied (Table 1), but they did not fall into such distinct categories. The minor diterpenes, isophyllocladene (**3**), sclarene (**7**) and abietatriene (**8**), were tentatively identified by their retention indices [31], but as the levels of these components rarely exceeded 5%, only the variations of laurenene, rimuene and phyllocladene with different factors will be considered. Diterpene hydrocarbons were not detected in bark or wood samples. Female cones from one tree were analysed and the diterpene levels were similar to those of the foliage.

The study of variation within a single population of *D. cupressinum* was conducted in the Otago Coast Forest, in the south-east of the South Island of New Zealand. Three adult rimu, with high, medium, and low laurenene levels, were climbed by various means and foliage samples were taken from different positions. The relative and absolute diterpene levels were quite uniform over each tree, and approximately normally distributed. For example the mean rimuene level in one tree was 77% of the total diterpenes, with a standard deviation of 3%. Subsequent samples were generally taken from the lowest, most accessible branches.

Table 1. Diterpene levels in 40 adult rimu

	% of diterpenes	
Identity*	Median	Range
?	0	0–3
Laurenene	34	0–97
Rimuene	47	1–94
Sclarene	1	0–13
?	1	0–3
Isophyllocladene	1	0–11
?	0	0–1
Phyllocladene	9	0–46
Abietatriene	0	0–1

*Retention index increases down the list.

Two of the rimu sampled at different heights were growing with their trunks pressed together and branches so intertwined that they were originally thought to be a single tree. These two trees obviously experienced the same environmental conditions of light, temperature, water, wind and soil, but had totally different levels of laurenene. In one tree the laurenene levels ranged from 4% to 24% while in the other they ranged from 36% to 52%. The rimuene levels were also distinct. Furthermore, the twenty trees sampled in the Otago Coast Forest showed ranges of diterpene levels as great as those met in samples from other regions. Thus environmental factors do not seem to influence diterpene levels.

To check for seasonal variation in diterpene levels, nine trees were sampled several times over a period of a year and a half. The compositional pattern in each tree varied only slightly, with no significant increases or decreases in the levels of laurenene, rimuene and phyllocladene between any two sampling times.

The diterpene levels in foliage samples collected on the same day from ten male and ten female trees were compared. Since these were not normally distributed (see for example Fig. 1), non-parametric statistical methods had to be applied. A suitable non-parametric test is the Kruskal–Wallis one-way analysis of variance [34, 35]. All the samples are ranked according to the value of the variable in question, then the rank sums of the different groups are tested for significant differences. None of the levels considered varied significantly between the male and female groups, so the diterpene hydrocarbons do not seem to be related to sexual expression.

Ten juvenile rimu (between one and three metres in height) were sampled on the same day as ten adult trees in the same area. The levels of 26 different GC peaks in the extracts of these two groups were compared by the Kruskal–Wallis test. Only the phyllocladene (**2**) peak

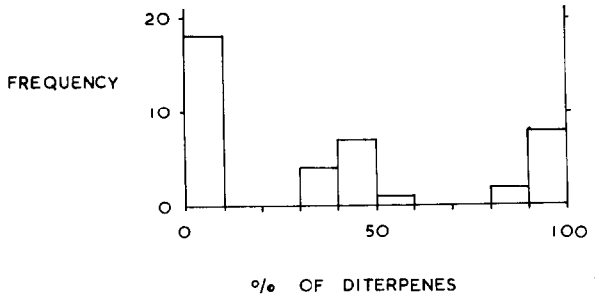


Fig. 1. Laurenene levels in 40 adult rimu.

differed significantly ($p < 0.01$) between adult and juvenile trees (Table 2).

Samples from two other rimu populations were examined: foliage was collected from ten adult trees near Hokitika on the West Coast of the South Island and from ten trees in Pureora Forest in the central North Island. The levels of the three major diterpenes in these samples were first compared by the Kruskal–Wallis test and highly significant ($p < 0.01$) differences were found (Table 3). Comparison of pairs of populations showed similar diterpene levels in the Otago Coast Forest and Pureora samples. Both of these samples differed significantly, in laurenene, rimuene and phyllocladene levels, from the Hokitika sample.

Analyses of the data from all 40 adult trees showed correlations between the levels of the three major diterpenes which will be discussed below.

DISCUSSION

Laurenene, rimuene and phyllocladene were the major diterpenes in all the samples of rimu foliage examined. Their levels were quite uniform in the foliage of a single tree over a period of time, but varied greatly between neighbouring trees. Thus, the variation does not seem to be due to any environmental factors and no seasonal variation was detected. This leads to the conclusion that the diterpene levels in rimu foliage are genetically controlled, and that the variation is another example of morphism ". . . in which (usually sharply distinct) genetic variants or morphs coexist in temporary or permanent balance within a single spatial region . . ." [36]. No controlled breeding programmes could be traced, so there is no direct evidence of genetic control of diterpene levels in rimu. However, similar conclusions were reached in two other studies of diterpene levels in conifer foliage.

Table 2. Phyllocladene levels in adult and juvenile rimu

	% of diterpenes		
Tree age	Median	Range	Rank sum
10 Adult	11.5	3–51	147.0
10 Juvenile	0.5	0–13	63.0

Kruskal–Wallis statistic = 10.172; $p = 0.0014$.

The infraspecific variation of diterpenes in the leaves of *Cryptomeria japonica* D. Don (Taxodiaceae) [29] has been shown to be under genetic control by breeding studies [37]. In this case, seasonal variation was found. The current year's growth of foliage was examined and the diterpene hydrocarbon content was found to increase as the leaves grew to maturity [38]. A concurrent decrease of the rimuene to phyllocladene ratio was noted. In our work on *D. cupressinum*, no distinction was made between immature (the current year's) foliage and mature (previous year's) foliage. The rimuene to phyllocladene ratio did not differ significantly between the sampling times.

Gref has reported the variation of isoabienol (**9**) in the needles of *Pinus sylvestris* L. (Pinaceae) [39]. Two distinct classes of tree were found. "In one of these, isoabienol constituted 0.6–1.0% of needle dry weight, whereas no trace of isoabienol was found in the other . . ." Isoabienol content did not vary significantly on a single tree, nor seasonally. Gref concludes that the occurrence of isoabienol is a genetically controlled all-or-nothing character. He also found a difference between populations, with the frequency of isoabienol occurrence being higher further north.

Geographic variation of the foliage diterpenes of *D. cupressinum* was highly apparent: the Hokitika sample contained significantly more high laurenene trees than did the Otago Coast Forest or Pureora samples. Geographically separated chemical (usually monoterpene) races of North American conifers have been explained in terms of the distribution of a species in earlier geological periods. For example, Adams found distinct races of *Juniperus scopulorum* Sarg. (Cupressaceae) close together in the Northwest [40]. This area was glaciated during the last Ice Age, and Adams suggested that these

Table 3. Diterpene levels in adult rimu from various geographical areas*

	Laurenene†			Rimuene‡			Phyllocladene§		
Tree provenance	Median	Range	Rank sum	Median	Range	Rank sum	Median	Range	Rank sum
20 Otago Coast	6.0	0–90	353.0	57.0	7–94	506.0	11.0	0.5–45	444.0
10 Pureora	3.0	0–94	148.0	49.8	2–76	217.0	19.3	0–37	283.5
10 Hokitika	93.0	3–96	319.0	2.7	1–81	97.0	0.6	0–17	92.5

*Expressed as % of total diterpenes.
†Kruskal–Wallis statistic = 13.080 ($p = 0.0014$).
‡Kruskal–Wallis statistic = 12.012 ($p = 0.0025$).
§Kruskal–Wallis statistic = 14.239 ($p = 0.0008$).

adjacent, but distinct, races were due to colonization from different populations that survived further south. The discovery of distinct races of *D. cupressinum* could give evidence of the course of reforestation of the South Island after the last glaciation [41], but further sampling would be needed.

Phyllocladene (**2**) levels were significantly higher in adult foliage than in juvenile foliage. The levels of phyllocladene, isophyllocladene (**3**) and kaurene (**10**) in juvenile foliage of *D. kirkii* F. Muell. have been reported to be lower than those in adult foliage, but only one specimen of each was examined [6]. Differences in levels of mono- and sesquiterpenes between adult and juvenile foliage have also been noted in *J. scopulorum* (Cupressaceae) [42]. We have also noted a significant difference between the diterpenes of adult and juvenile foliage from *D. biforme* (Hook.) Pilger and this will be reported in a subsequent paper.

Strong negative correlation between laurenene and rimuene levels, expressed as percentages of the total diterpenes, was observed. This is not surprising, as these are the major diterpenes in all but one of the 40 trees.

This artificial correlation was avoided by using levels relative to the internal standard, which are proportional to the absolute amount of each component in an extract. The Kendall rank coefficient was used as a measure of correlation since the variables were not normally distributed, and to check for significant interdependences of the levels of the major diterpenes [34, 35]. Laurenene levels showed significant negative correlations with the levels of both rimuene and phyllocladene (the rank coefficients were -0.343 and -0.405 respectively), whilst these latter components showed a significant positive correlation (rank correlation co-efficient of 0.472). For this data, the critical value of the rank correlation coefficient for $p < 0.01$ was 0.285. Zavarin has suggested that such correlations in terpene levels must be due to "... linkage of biosynthetic reaction sequences", and gives several monoterpene examples [43]. Using his terms, laurenene is in substitutional relationships with both rimuene and phyllocladene, and these last two are in proportional relationship. Rimuene (**1**) and phyllocladene (**2**) are both 'regular' polycyclic diterpenes, thought to be derived from a common bicyclic intermediate [44]. Although the biosynthetic pathway to laurenene (**4**) is not known, it clearly does not share any immediate precursor with the conventional diterpenes. The substitutional relationship between laurenene and both rimuene and phyllocladene could be due to there being only a limited 'pool' of geranylgeranyl pyrophosphate available for diterpene synthesis, so that the enzyme (or enzymes) synthesizing laurenene compete for this substrate with the enzyme(s) synthesising rimuene and phyllocladene.

No compounds that could represent intermediates in the biosynthesis of laurenene were identified.

During this study it was noted that the levels of the foliage sesquiterpenes of *D. cupressinum* also varied considerably. The identities of these components and the factors affecting this variation will be reported in a subsequent paper.

The generic boundaries in the family Podocarpaceae are under scrutiny by taxonomists [45, 46] and an attempt has been made to use foliage diterpenes as taxonomic characters [6]. However, this study ignored the basic principle that the variability of any characters be they morphological, anatomical or chemical, must be assessed before taxonomic conclusions may be drawn [30, 47, 48]. The variations of foliage components of other New Zealand gymnosperms are now being studied.

EXPERIMENTAL

Sampling. Rimu trees, identified by the authors and by members of the New Zealand Forest Service, were assigned individual numbers and their locations were recorded. Voucher specimens have been deposited with the Otago University Herbarium, but sample numbers are not yet available. Sampling details and the diterpene composition of each extract have been submitted with this manuscript.* Foliage samples were stored at *ca* 5° and were extracted within a day of collection when possible.

Extraction. Terminal twigs and leaves were ground under liquid N_2. The resulting material (5 g), suspended in H_2O (150 ml) was steam-distilled/solvent-extracted [49] for 2 hr into pentane (10 ml), which contained *n*-octadecane (1.0 mg) as an internal standard.

GC analyses. Extracts were analysed on a 15 m SE-30 WCOT fused-silica column without further concentration. Injections were 0.05–1.0 μl with a split ratio of *ca* 1:50. The injector temp. was 230° and the FID temp. was 240°. The carrier gas was He with a linear velocity of *ca* 50 cm/sec. The column temp. was programmed from 100° to 200° at 8°/min. Peak areas and R_ts were measured with an electronic integrator. Laurenene, rimuene and phyllocladene peaks were identified by their highly consistent RR_ts [31]. Relative molar responses of the FID for these three compounds (measured relative to that of *n*-octadecane, with standard errors in brackets) are laurenene 0.63 (0.004); rimuene 0.34 (0.002); phyllocladene 0.45 (0.01).

Data handling. Peak identities and areas were entered into a computer interactively, and processed (listings of Fortran programs are available), prior to analysis, with a standard statistical package [34].

Acknowledgements—The New Zealand Forest Service gave essential assistance with sampling. Dr. Wayne Sutherland provided GC equipment and Dr. Brian Niven advised on statistical analysis. Grants from the Research Committee of the University of Otago and from the University Grants Committee are gratefully acknowledged.

REFERENCES

1. Enting, B. L. and Molloy, L. (1982) *The Ancient Islands.* Port Nicholson Press, Wellington.
2. Kelly, G. C. (1980) in *Land Alone Endures: Land Uses and The Role of Research* (Molloy, L. F., ed.) p. 83. Science Information Division, DSIR, Wellington.
3. Allan, H. H. (1961) *Flora of New Zealand.* New Zealand Government Printer, Wellington.
4. Salmon, J. T. (1980) *The Native Trees of New Zealand.* A.H. & A.W. Reed, Wellington.
5. Florin, R. (1963) *Acta Hortic. Berg.* **20**, 122.
6. Aplin, R. T., Cambie, R. C. and Rutledge, P. S. (1963) *Phytochemistry* **2**, 205.
7. Cambie, R. C. (1976) *J. Roy. Soc. N. Z.* **6**, 307.
8. Franklin, D. A. (1968) *N. Z. J. Botany* **6**, 493.
9. McDowall, F. H. and Finlay, H. J. (1925) *J. Soc. Chem. Ind.* **44**, 42T.
10. Connolly, J. D., McCrindle, R., Murray, R. D. H. and

*Details available from the authors.

Overton, K. H. (1966) *J. Chem. Soc. C* 273.
11. Corbett, R. E. and Wyllie, S. G. (1966) *J. Chem. Soc. C* 1737.
12. Briggs, L. H. (1937) *J. Chem. Soc.* 79.
13. Sasse, J. M., Rowan, K. S. and Galbraith, M. N. (1981) *Phytochemistry* **20**, 2195.
14. Singh, P., Fenemore, P. G., Dugdale, J. S. and Russell, G. B. (1978) *Biochem. Syst. Ecol.* **6**, 103.
15. Corbett, R. E., Lauren, D. R. and Weavers, R. T. (1979) *J. Chem. Soc. Perkin Trans.* 1, 1774.
16. Corbett, R. E., Couldwell, C. M., Lauren, D. R. and Weavers, R. T. (1979) *J. Chem. Soc. Perkin Trans.* 1, 1791.
17. Greenberg, A. and Liebman, J. F. (1978) *Strained Organic Molecules*, p. 373. Academic Press, New York.
18. Paquette, L. A. (1984) *Topics in Current Chemistry* **119**, 84.
19. Bohlmann, F. and Jakupovic, J. (1980) *Phytochemistry* **19**, 259.
20. Kaneda, M., Takahashi, R., Iitaka, Y. and Shibata, S. (1972) *Tetrahedron Letters* 4609.
21. Eaton, P. J., Fawcett, J. M., Jogia, M. K. and Weavers, R. T. (1980) *Aust. J. Chem.* **33**, 371.
22. Nathu, N. K. and Weavers, R. T. (1980) *Aust. J. Chem.* **33**, 1589.
23. Eaton, P. J., Lauren, D. R., O'Connor, A. W. and Weavers, R. T. (1981) *Aust. J. Chem.* **34**, 1303.
24. Corbett, R. E., Jogia, M. K., Lauren, D. R. and Weavers, R. T. (1983) *Aust. J. Chem.* **36**, 1001.
25. Hanton, L. R., Simpson, J. and Weavers, R. T. (1983) *Aust. J. Chem.* **36**, 2581.
26. Mabry, T. J. (1973) *Pure Appl. Chem.* **34**, 377.
27. Tetenyi, P. (1970) *Infraspecific Chemical Taxa of Medicinal Plants*. Chemical Publishing Co., New York.
28. Carman, R. M. and Sutherland, M. D. (1979) *Aust. J. Chem.* **32**, 1131.
29. Appleton, R. A., McCrindle, R. and Overton, K. H. (1970) *Phytochemistry* **9**, 581.
30. Davis, P. H. and Heywood, V. H. (1963) *Principles of Angiosperm Taxonomy*. Oliver & Boyd, Edinburgh.
31. Perry, N. B. and Weavers, R. T. (1984) *J. Chromatogr.* **284**, 478.
32. Von Rudloff, E. (1974) *Adv. Chromatogr.* **10**, 173.
33. Novak, J. (1974) *Adv. Chromatogr.* **11**, 1.
34. Dixon, W. J. (ed.) (1981) *BMDP Statistical Software*, p. 437. University of California Press, Berkeley.
35. Sokal, R. R. and Rohlf, F. J. (1981) *Biometry*, 2nd edn. W. H. Freeman, San Francisco.
36. Huxley, J. S. (1955) *Heredity* **9**, 1.
37. Yashue, M., Ogiyama, K., Ebishigawa, S., Kondo, K. and Nishina, K. (1978) *J. Jap. For. Soc.* **60**, 345.
38. Kawashima, T., Yashue, M. and Ogiyama, K. (1978) *J. Jap. For. Soc. (Tohoku District)* **29**, 112.
39. Gref, R. (1981) *Can. J. Botany* **59**, 831.
40. Adams, R. P. (1983) *Taxon* **32**, 30.
41. Fleming, C. A. (1979) *The Geological History of New Zealand and Its Life*, p. 71. Auckland University Press, Auckland.
42. Adams, R. P. and Hagerman, A. (1976) *Biochem. Syst. Ecol.* **4**, 75.
43. Zavarin, E. (1970) *Phytochemistry* **9**, 1049.
44. West, C. A. (1981) in *Biosynthesis of Isoprenoid Compounds* (Porter, J. W. and Spurgeon, S. L., eds) Vol. 1, p. 375. Wiley, New York.
45. De Laubenfels, D. J. (1969) *J. Arnold Arbor. Harv. Univ.* **50**, 274.
46. Quinn, C. J. (1982) *Aust. J. Botany* **30**, 311.
47. Stace, C. A. (1980) *Plant Taxonomy and Biosystematics*. Edward Arnold, London.
48. Siegler, D. S. (1981) in *Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds) Vol. 7, p. 139. Academic Press, New York.
49. Likens, S. T. and Nickerson, G. B. (1964) *Am. Soc. Brewing Chem. Proc.* 5.