ELSEVIER

Journal of Chromatography A, 715 (1995) 317–324

JOURNAL OF CHROMATOGRAPHY A

Gas chromatographic–mass spectrometric characterization of some fatty acids from white and interior spruce☆

Danielle-Julie Carrier, James E. Cunningham, Lawrence R. Hogge, David C. Taylor, David I. Dunstan*

Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Sask. S7N OW9, Canada

First received 28 March 1995; revised manuscript received 19 May 1995; accepted 19 May 1995

Abstract

The objective of this work was to determine the fatty acid composition of white and interior spruce seeds. Fatty acid methyl ester derivatives obtained from the seed oils were analyzed by gas chromatography. Elution times for some of the spruce fatty acid methyl ester derivatives did not correspond to those of available standards. Diethylamide derivatives were prepared and analyzed by gas chromatography–mass spectrometry. The electron-impact mass spectral fragmentation patterns of the fatty acids of interest indicated *cis-11*-18:1, *cis-5*,*cis-9*-18:2 and *cis-5*,*cis-9*,*cis-12*-18:3.

1. Introduction

Interior spruce (*Picea glauca engelmannii* complex) is a natural hybrid between white (*Picea glauca*) and Engelmann (*Picea engelmannii*) spruces that occurs where their ranges overlap. It is an economically important species in British Columbia where 80 million interior spruce seedlings are planted annually. Our goal is to facilitate the production of genetically improved spruce through the development of a clonal propagation system by in vitro formation of spruce embryos. Synthetic seed production, involving an artificial endosperm (i.e. storage reserve for the germinating seedling), is a component of this goal. To develop an artificial endosperm, a better understanding of the nutritional requirements of germinating spruce seedlings would provide a useful basis for our investigation.

Spruce seeds contain approximately 30% lipid by weight [1]. This high lipid concentration suggests that lipid metabolism is, as in other gymnosperms [2], important in the supply of energy during germination before the seedling gains autotrophy. As part of our study, we investigated the lipid content and composition of interior spruce seeds. To our knowledge, no study on the fatty acid content of seeds of this species has been reported. In preliminary gas chromatographic (GC) investigations with transmethylated products from interior spruce oils,

* Corresponding author.

☆ National Research Council of Canada Publication No. 38920.

SSDI 0021-9673(95)00601-X

the second most abundant fatty acid methyl ester (FAME) within the profile was difficult to identify by matching with standards. This peak eluted between *cis-9,cis-12*-18:2 and *cis-9,cis-12,cis-15*-18:3 FAME derivatives. A preliminary gas chromatographic–mass spectrometric (GC–MS) characterization indicated that the molecular ion of this peak corresponded to that of an 18:3 FAME. In a previous study on the fatty acids contained in the closely related white spruce [1], the second most abundant component was reported to be *5,9*-18:2. To clarify and advance the record of spruce seed fatty acid composition, we undertook the identification of the major fatty acids of white and interior spruce seeds.

Many methods are available for characterizing unsaturated aliphatic compounds by GC–MS. Formation of vicinal diols followed by the formation of either acetonides, boronates, as well as silyl and methyl ethers are common procedures for double-bond elucidation [3]. Although the resulting mass spectral data are informative, the use of osmium tetroxide in the reaction is potentially hazardous. The position of double bonds can also be determined by epoxidation with *m*-chloroperbenzoic acid followed by hydrogenation [3]. Although a successful method, the two-step derivatization procedure is time-consuming. The incorporation of a charge-stabilizing group, such as an amide, at the carboxyl group is another useful method in locating or determining the position of double bonds [4]. The location of the double bond can be deduced from the fragmentation patterns of the mass spectra. Pyrrolidine is generally recommended as the amine of choice for mass spectrometric elucidation of fatty acid structure [3]. However, when the fatty acid in question contains no hydroxy, epoxy or other charge retaining groups, a diethylamine derivatization can be utilized [4]. The advantage of this method, over others, is the ease of derivatization and the simplicity of the resulting mass spectrum. This method was successfully used to determine the double-bond positions of unsaturated fatty acids of Norway spruce (*Picea abies*) [5].

We present here the results of our investigation into the identity of the major fatty acids contained in lipid extracts of white and interior seeds. The lipid extracts were prepared, derivatized into FAMEs and analyzed by GC. Further characterization of the fatty acids involved derivatization with diethylamine and GC–MS analysis.

2. Experimental

2.1. Chemicals

All chemicals were reagent grade. Methanolic HCl (3 *M*) was purchased from Supelco Canada (Oakville, Ont., Canada). Diethylamine and glacial acetic acid were obtained from Aldrich (Milwaukee, WI, USA) and Fisher Scientific (Nepean, Ont., Canada), respectively. *Picea glauca* (white spruce) and *Picea glauca engelmannii* complex (interior spruce) seeds were obtained from Prairie Farm Rehabilitation Administration (Indian Head, Sask., Canada) and British Columbia Research (Vancouver, BC, Canada), respectively. Heptadecanoic acid (C17:0) and other FAME standards were purchased from Nu-Chek-Prep (Elysian, MN, USA).

2.2. Methods

Initial methyl ester study

Interior spruce seeds were extracted and methylated according to published procedures [6–8]. The fatty acid 17:0 was added as an internal standard. FAMEs were analyzed as described previously [8].

Gas chromatography–mass spectrometry

All GC–MS analyses were performed using a Fisons 8000 gas chromatograph (Fisons Instruments, Manchester, UK) which was fitted with a 60 m × 0.32 mm I.D. DB-23 fused-silica column (J&W Scientific, Folsom, CA, USA) and interfaced to a Fisons Tri 2000 quadrupole mass spectrometer. All samples were injected using the split injection mode. The initial column temperature was 70°C and was ramped at a rate of 20°C per min to 180°C, followed by a programmed increase of 4°C per min to 240°C. The GC interface and source were maintained at

250°C. Repetitive scans were taken every 1.1 s in the mass range of 50 to 510. The electron energy was 70 eV.

Total lipid extraction and diethylamide derivatization

Isopropanol (1.5 ml) was added to 100 mg of seed and the mixture was homogenized with an Ultra-Turrax (Janke & Kunkel, Germany) for 3 min at maximum speed. The mixture was capped and plunged into a boiling water bath for 5 min. After cooling, 0.75 ml CH_2Cl_2 was added and the mixture was incubated for 30 min at room temperature with occasional vortexing. Following incubation, 1 ml water and 2 ml CH_2Cl_2 were added. The mixture was vortexed and centrifuged at 830 *g*. The organic phase was retained and the aqueous phase re-extracted twice with 2 ml CH_2Cl_2. The organic phases were combined and the solvent was evaporated to yield the total lipid extract. To obtain diethylamide derivatives, the protocol devised in Ref. [5] was essentially followed. The total lipid extract was transferred to a 1-ml Pierce Reacti Vial (Rockford, IL, USA), to which 0.8 ml diethylamine and 0.1 ml of glacial acetic acid were added. The vial was purged with N_2, tightly sealed and placed in a Pierce Reacti-Therm (Rockford, IL, USA) at 105°C for 75 min. Afterwards, the mixture was cooled and transferred to a screw-cap glass test tube. The diethylamine was evaporated under a stream of N_2. To this tube, 1 ml water and 2 ml $CHCl_3$ were added, vortexed and centrifuged at 830 *g*. The organic phase, containing the diethylamide derivatives, was retrieved and evaporated to dryness.

3. Results and discussion

3.1. Methyl ester derivatization

The direct methylation method [6] yielded 150 μg of total lipid fatty acids per mg fresh weight of seed. This method does not always give quantitative recovery of fatty acids from all plant tissues. It works well for leaf tissue, as used originally [6], but not necessarily for other tissues, such as seeds of interior spruce. The total lipid extraction procedures described by Hara and Radin [7] and Holbrook et al. [8], followed by methylation and GC analysis, yielded amounts in the range of 300 μg of total lipid fatty acids per mg fresh weight of seed. All of the following extractions and transmethylations used the methods described by Holbrook et al. [8].

The results of GC–MS analysis of the total lipid extract of interior spruce seeds are shown in Fig. 1. Peaks 1, 2, 3 and 6 were identified as 16:0, 18:0, *9*-18:1 and *9,12*-18:2 FAMEs, respectively, by comparison to the retention times and mass spectral data of standards. Under the current chromatographic conditions, *trans-9*-18:1 and *trans-9,trans-12*-18:2 FAMEs eluted about 0.5 min earlier than the corresponding *cis* isomers: *cis-9*-18:1 and *cis-9,cis-12*-18:2 FAMEs. By combining this with the fact that lipids of plant origin are typically of *cis* configuration, it was inferred that the FAMEs detected in this work were also of *cis* configuration. Hence, peaks 3 and 6 of Fig. 1. were identified as *cis-9*-18:1 and *cis-9,cis-12*-18:2 FAMEs.

Mass spectral data from the second largest component in Fig. 1 (labelled number 7) indicated a molecular ion of 292 which corresponds to an 18:3 FAME. However, the retention time did not correspond to any available standards. Similarily, component number 5 showed a molecular ion of 294, indicating an 18:2 FAME of unknown double-bond positional configuration.

The results of GC–MS analysis of the total lipid extract of white spruce seeds are shown in Fig. 2. Similarity between the FAME profiles of the two species was observed. The molecular ions of peaks D and E were 296 and 294, which indicated 18:1 and 18:2 methyl esters, respectively. Elucidation of the structures of the compounds corresponding to peaks 5 and 7 of Fig. 1 and to peaks D and E of Fig. 2 is presented below.

3.2. Diethylamide derivatization

Diethylamide derivatization provides the analyte with a charge stabilizing group which retards rearrangement of the molecular ion before fragmentation occurs [3]. This method was first

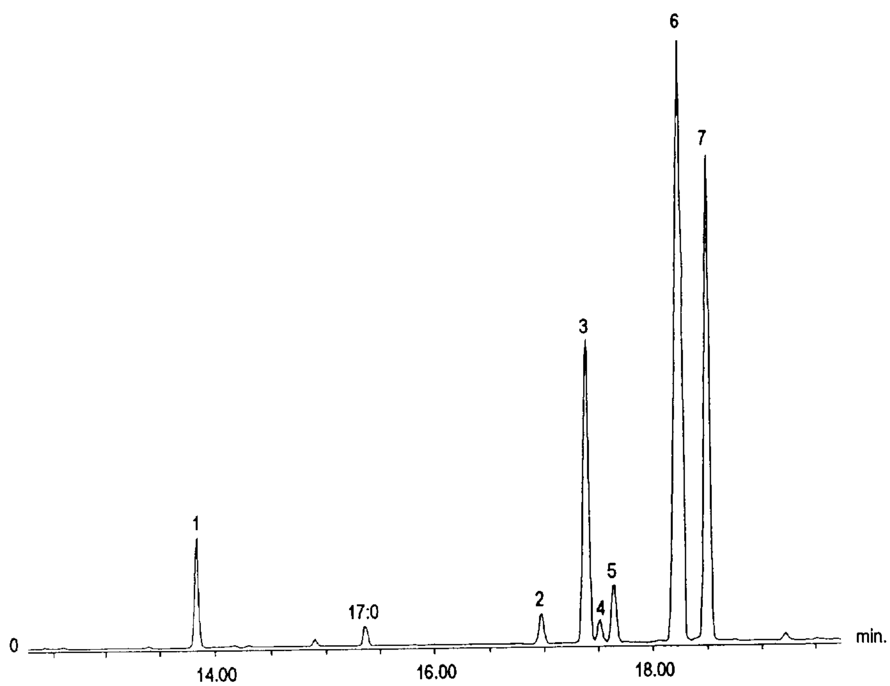

Fig. 1. Mass chromatogram of FAMEs obtained from interior spruce oil extract.

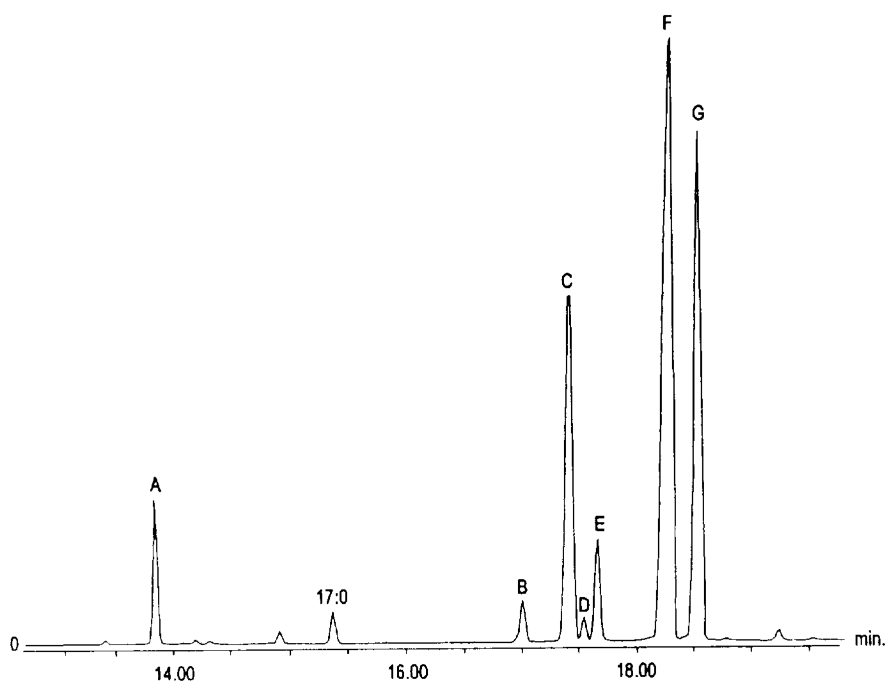

Fig. 2. Mass chromatogram of FAMEs obtained from white spruce oil extract.

assessed with a *cis-9,cis-12,cis-15*-18:3 (α-linolenic acid) as a reference compound. The resulting mass spectrum was interpreted using the rules presented in Ref. [5], where saturated bonds are represented by fragments separated by 14 mass units. Fragments separated by 12 mass units between carbons n and $n+1$ indicate the presence of a double bond between carbons $n+1$ and $n+2$. The mass spectrum of the diethylamide derivative of *cis-9,cis-12,cis-15*-18:3 was readily interpreted using these rules. This mass spectrum was similar to that reported in Ref. [5].

The electron-impact mass spectral fragmentation patterns of diethylamide derivatives of the compounds, corresponding to peaks 6 and 7 of Fig. 1, are shown in Figs. 3A and B, respectively. The mass spectrum shown in Fig. 3A displays a molecular ion of 335 mass units, corresponding to a diethylamide derivative of 18:2. Differences of 12 mass units between fragments m/z 198–210 and m/z 238–250, ascribed double-bond locations to carbons 9–10 and 12–13, respectively, identifying the compound as *cis-9,cis-12*-18:2, as reported in Ref. [5]. The mass spectrum of the diethylamide derivative of the second most abundant fatty acid is shown in Fig. 3B. This compound appeared to have a molecular ion of 333 mass units, indicating a diethylamide derivative of 18:3. Differences of 12 mass units were observed between the fragment ions at m/z 142–154, 196–208 and 236–248, indicating double bonds in the 5, 9 and 12 positions. Taking into consideration that, in spruce, *9,12*-18:2 was of *cis* configuration, the compound was identified as *cis-5,cis-9,cis-12*-18:3.

The electron-impact mass spectral fragmentation pattern of the diethylamide derivative of the compound corresponding to peak 5 of Fig. 1 was not sufficiently intense to assign double-bond locations. However, the mass spectral fragmentation pattern of the white spruce diethylamide derivative corresponding to peak E of Fig. 2 was sufficiently intense that double-bond locations could be identified. Results are presented in Fig. 4A, where a molecular ion of 335 mass units, indicating a diethylamide derivative of 18:2, was observed. The fragmentation pattern indicated double bonds in the 5 and 9 position, identifying the compound as *cis-5,cis-9*-18:2. Fig. 4B shows the diethylamide derivative fragment spectrum of the compound, corresponding to peak D of Fig. 2. The molecular ion of 337 mass units corresponded to that of an 18:1 diethylamide derivative. Although less clear, the mass spectrum of this compound showed a 12 mass unit gap between masses 226–238; indicating the double bond to be on carbons 11 and 12. This compound was tentatively identified as *cis-11*-18:1.

In comparing the retention times of FAMEs obtained from white spruce to those of interior spruce, it was inferred that peak 5 of Fig. 1 and peak E of Fig. 2 were equivalent (i.e. both are believed to be *cis-5,cis-9*-18:2). Similarly, it was observed that peak D of Fig. 2 and peak 4 shown in Fig. 1 had similar retention times. Hence, they were both tentatively identified as *cis-11*-18:1.

The FAMEs corresponding to peaks A, B, C, D, E, F and G of Fig. 2 were identified as 16:0, 18:0, *cis-9*-18:1, *cis-11*-18:1, *cis-5,cis-9*-18:2, *cis-9,cis-12*-18:2 and *cis-5,cis-9,cis-12*-18:3. The distribution of these fatty acids, found in white and interior spruce seeds, is shown in Table 1. White and interior spruce seed lipid contents were 49 ± 5% and 41 ± 1% lipid by fresh weight, respectively.

The spectra of the *cis-5,cis-9*-18:2 and *cis-5,cis-9,cis-12*-18:3 diethylamide derivatives each have an intense ion at m/z 182 (relative to the other aliphatic chain cleavage ions). The intensity of this ion is likely due to the allylic fragments formed when there are two methylene groups separating the double bonds. The intensity of this ion makes it diagnostically useful in determining this unusual double-bond configuration. This postulation is borne out by examining the spectra of the fatty acid derivatives shown in Figs. 3B and 4A. The spectra shown in Figs. 3A and 4B do not display an intense ion at m/z 182.

The fatty acid *cis-5,cis-9,cis-12*-18:3 was detected in *P. abies* [5,9,11] and in *P. engelmannii, mariana, obovata, orientalis* and *sitchensis* [10]. Our results confirm this finding for *P. glauca* and *P. glauca engelmannii* complex. Other workers [1,12] reported the second most abundant fatty acid in *P. glauca* to be *cis-5,cis-9*-18:2. The *P.*

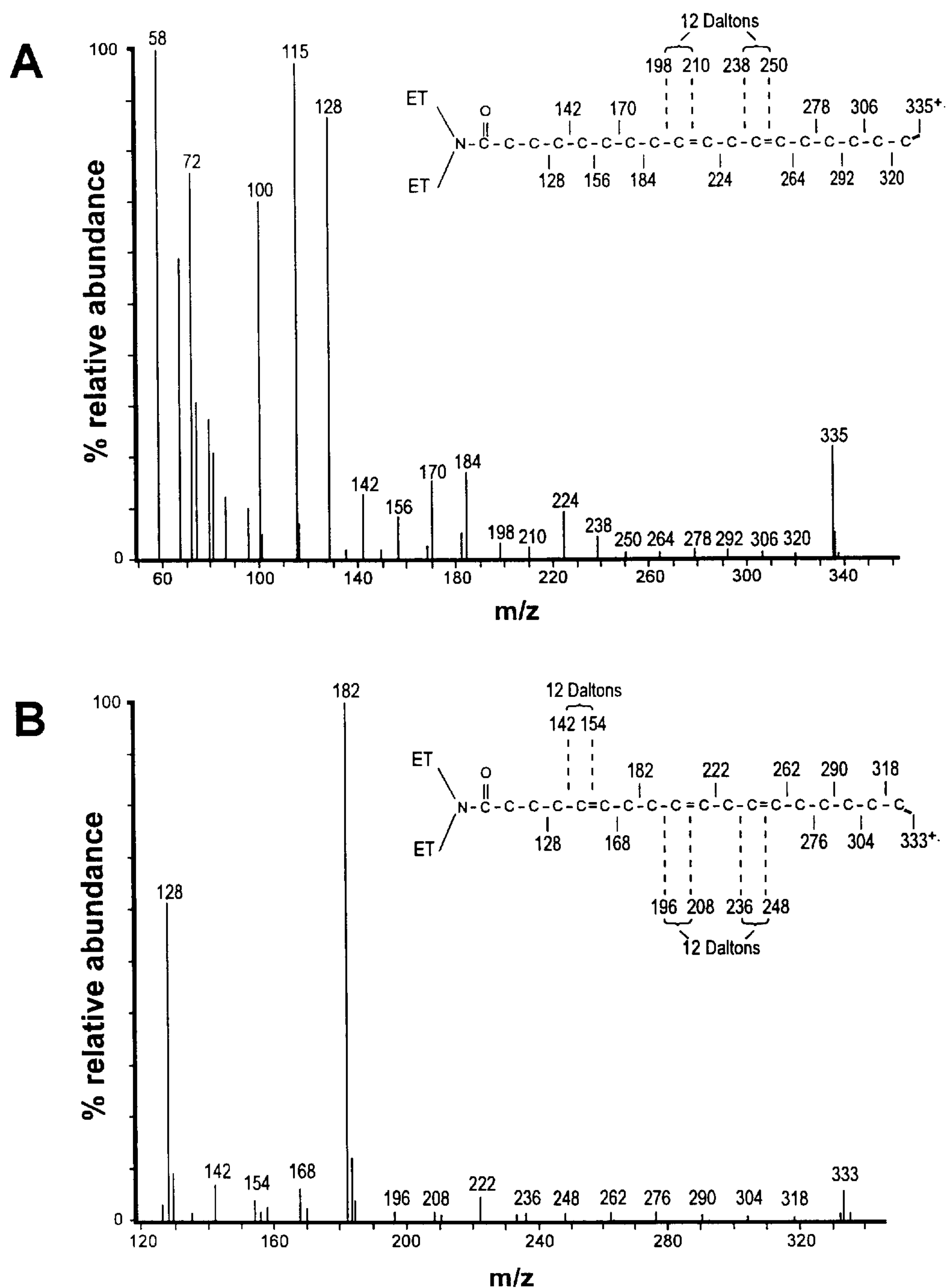

Fig. 3. Mass spectrum of diethylamide derivatives of interior spruce. (A) Mass spectrum of *cis-9,cis-12*-18:2. (B) Mass spectrum of *cis-5,cis-9,cis-12*-18:3. The compounds used to obtain these diethylamide derivatives are identical to the ones used to obtain the FAME derivatives corresponding to peaks 5 and 6, respectively, presented in Fig. 1.

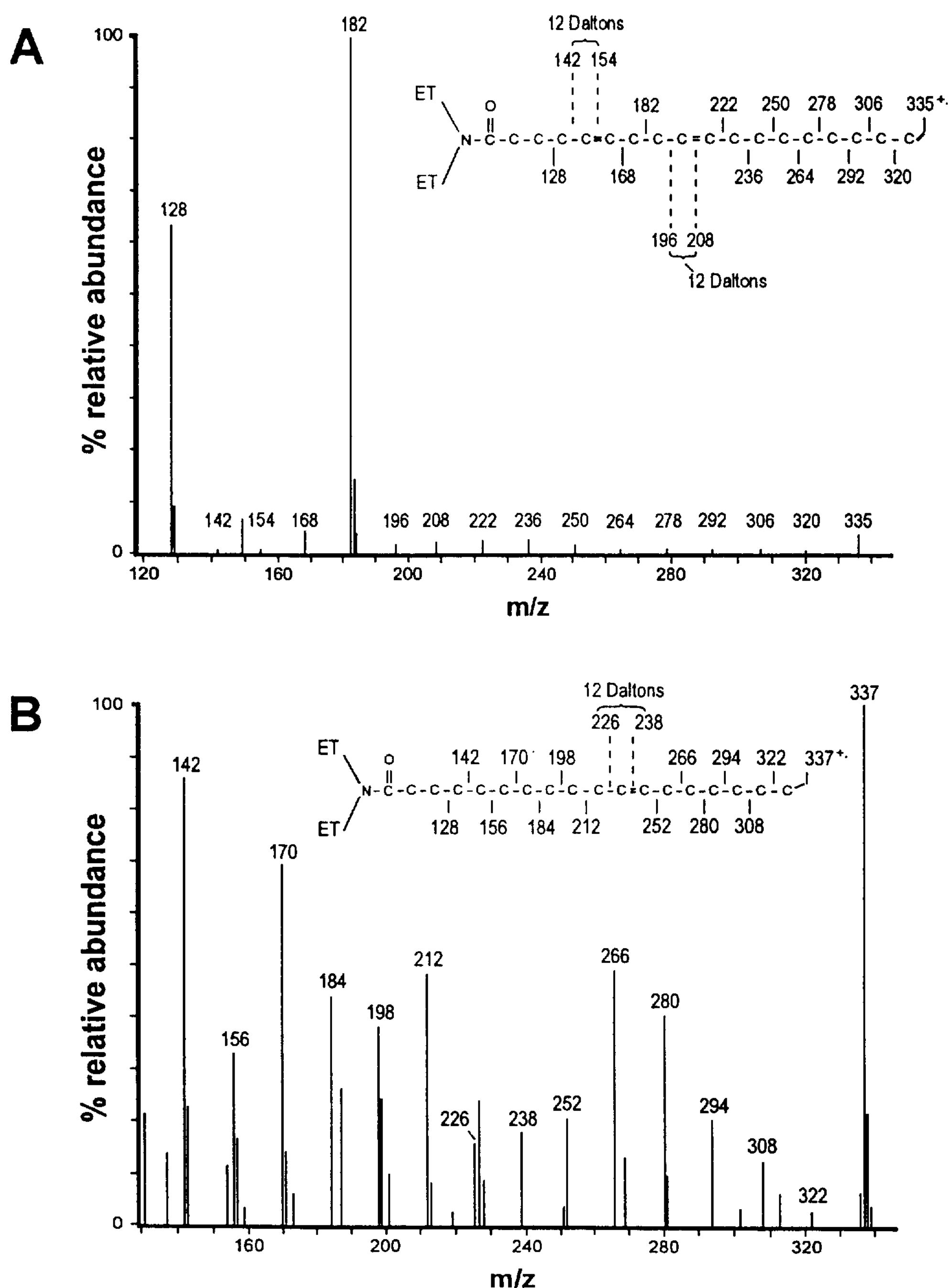

Fig. 4. Mass spectrum of diethylamide derivatives of white spruce. (A) Mass spectrum of *cis-5,cis-9*-18:2. (B) Mass spectrum of *cis-11*-18:1. The compounds used to obtain these diethylamide derivatives are identical to the ones used to obtain the FAME derivatives corresponding to peaks E and D, respectively, presented in Fig. 2.

Table 1
Distribution of 16:0, 18:0, *cis-9*-18:1, *cis-11*-18:1, *cis-5,cis-9*-18:2, *cis-9,cis-12*-18:2 and *cis-5,cis-9,cis-12*-18:3 methyl esters in white and interior spruce expressed in μg per mg of tissue and in percentage of oil content

Seed type	16:0	18:0	9-18:1	11-18:1	5,9-18:2	9,12-18:2	5,9,12-18:3
White spruce							
$\mu g/mg$	12 ± 2	6 ± 1	74 ± 13	5 ± 1	15 ± 2	209 ± 33	103 ± 21
% of oil content	3	2	17	1	4	49	24
Interior spruce							
$\mu g/mg$	10 ± 1	7 ± 1	74 ± 11	6 ± 1	13 ± 3	196 ± 23	108 ± 12
% of oil content	3	2	18	1	3	47	26

glauca seeds extracted in our laboratory did contain this fatty acid. However, it was determined to be a minor component of the oil, as shown in Table 1.

Acknowledgements

We thank British Columbia Research (Vancouver, BC, Canada) and Dan Walker of Prairie Farm Rehabilition Administration (Indian Head, Sask., Canada) for providing us with interior and white spruce seed, respectively. The authors wish to thank Mike Giblin, Darwin Reed and Douglas Olson for expert technical assistance. This work has been funded by the National Research Council of Canada Agreement, GC103-3-2021, by technology contribution between by the National Research Council of Canada and British Columbia Research.

References

[1] S.M. Attree, M.K. Pomeroy and L.C. Fowke, Planta, 187 (1992) 395.
[2] T.M. Ching, in T.T. Kozlowski (Editor), Seed Biology, Vol. II, Academic Press, New York, 1972, p. 103.
[3] L. Hogge and J. Millar, in J.C. Giddings et al. (Editors), Advances in Chromatography, Vol. 27, Marcel Dekker, New York, 1987, p. 299.
[4] B.A. Andersson, W.H. Heimermann and R.T. Holman, Lipids, 9 (1974) 443.
[5] R. Nilsson and C. Liljenberg, Phytochem. Anal., 2 (1991) 253.
[6] J. Browse, P.J. McCourt and C.R. Somerville, Anal. Biochem., 152 (1986) 141.
[7] A. Hara and N.S. Radin, Anal. Biochem., 90 (1978) 420.
[8] L.A. Holbrook, J.R. Magus and D.C. Taylor, Plant Sci., 84 (1992) 99.
[9] R. Ekman, Phytochemistry, 19 (1980) 147.
[10] G.R. Jamieson and E.H Reid, Phytochemistry, 11 (1972) 269.
[11] M. Olsson, R. Nilsson, P. Norberg, S. von Arnold and C. Liljenberg, Plant Physiol. Biochem., 32 (1994) 225.
[12] S.M. Attree, M.K. Pomeroy and L.C. Fowke, Plant Cell Rep., 13 (1994) 601.