Phytochemistry, Vol. 26, No. 4, pp. 1045–1047, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

LIPID COMPOSITION OF *AZOLLA CAROLINIANA* BIOMASS AND ITS SEASONAL VARIATION

CELSO PAOLETTI, FABIO BOCCI, GIOVANNI LERCKER, POMPEO CAPELLA and RICCARDO MATERASSI

Centro di Studio dei Microorganismi Autotrofi del C.N.R., c/o Istituto di Microbiologia Agraria e Tecnica della Università degli Studi, 50100 Florence, Italy; *Dept. Protezione e Valorizzazione Agroalimentare, Sez. di Chimica e Tecnologia degli Alimenti, Università degli Studi, 40126 Bologna, Italy

(*Revised received* 30 *July* 1986)

Key Word Index—*Azolla caroliniana*; Azollaceae; biomass; lipids; fatty acids; unsaponifiable; sterols; alcohols.

Abstract—The chemical composition of the lipid fraction of the aquatic fern, *Azolla caroliniana*, grown in outdoor mass culture in Florence, was investigated. Palmitic acid was the main fatty acid, particularly in summer when it reached 51.6% of the total fatty acids. In autumn a decrease in palmitic acid content and an increase in α-linolenic acid content occurred. The percentage of unsaturated fatty acids changed from 36.1% in summer to 54% in the biomass harvested in November.

INTRODUCTION

Azolla is an aquatic fern harbouring the endosymbiotic heterocystous cyanobacterium *Anabaena azollae*. This N_2-fixing symbiosis has been extensively exploited for centuries in Asiatic tropical and temperate areas as a green manure for rice cultivation. Therefore it is expected that *Azolla* can be a major source of fixed N in paddy ecosystems also in Europe [1].

In relation to this problem, some years ago in the Research Centre on Autotrophic Microorganisms an investigation was undertaken on the mass culture of some *Azolla* species under the climatic conditions of Italy. It has been shown that the N fixation potential is high and that the biomass, introduced in the soil as green manure, gets rapidly decomposed [2–6]. The protein fraction amounts to 21–24% of the dry matter and it is characterized by a balanced amino acid composition [1]. Thus, another possible application of *Azolla* biomass could be as an animal feedstuff.

In the present paper the results of an investigation into the chemical composition of the lipid fraction of *A. caroliniana* biomass are reported.

RESULTS AND DISCUSSION

In Table 1 the data on total lipid and unsaponifiable content of *Azolla caroliniana* biomass harvested in different seasons are shown. Total lipid content was *ca* 14% of the dry matter. The total lipid content varies with the harvest period, increasing gradually as the season become cooler. On a dry wt basis the unsaponifiable fraction increases, but less regularly than the total lipids. In sample C there was a considerable increase in the proportion of the unsaponifiable fraction.

Since *Azolla* leaves harbour an appreciable amount of *Anabaena* trichomes, it seemed appropriate to include in Table 1 the data on total lipids and the unsaponifiable fraction typical of *Anabaena* species grown in outdoor culture in Florence. The lipid content of *Azolla* biomass is of the same order of magnitude as that of the cyanobacterial biomass.

Table 1. Lipid and unsaponifiable contents of *Azolla caroliniana* collected in three different periods of the year

Season of harvest Sample*	Lipids (% dry matter)	Unsaponifiable (% dry matter)	(% lipids)
A	12.7	1.3	10.2
B	14.3	1.4	9.8
C	16.4	2.0	12.2
Anabaena spp.	12.0	1.1	11.2

*The three samples of *Azolla* biomass represent the harvests made in the periods 15 July–15 August (sample A), 15 September–15 October (sample B) and 15 November–15 December (sample C).

The quantitative composition of fatty acids, reported in Table 2, is characterized by a high content of palmitic, linolenic and lignoceric acids. The latter, to our knowledge, is contained in vegetable fats in high amounts only in *Adenanthera pavonina* seeds. The presence of lignoceric acid has been reported in the terrestrial fern *Onoclea sensibilis* [7], in which the main component is linolenic acid, while in *Azolla* palmitic acid is the predominant component. Palmitic acid is also the major fatty acid of the symbiont *Anabaena*.

The presence in *Azolla* biomass of arachidonic acid, an intermediate in the synthesis of prostaglandins, is a discovery of interest; the amount found is lower than that reported for terrestrial ferns [7], but it is important to note that the content of this acid increases considerably in autumn.

The fatty acid composition shows quantitative variations, according to season. When the temperature falls an

Table 2. Variation in the fatty acid composition of *Azolla caroliniana* biomass (% of total fatty acids) in three periods of the year

Fatty acids	Sample* A	B	C
Capric	tr	tr	tr
Lauric	0.7	0.3	0.3
Myristic	0.5	0.3	0.4
n-Pentadecanoic	0.5	0.4	0.3
Palmitic	51.6	38.8	36.6
Palmitoleic	2.1	2.4	2.1
n-Hexadecanoic	1.7	1.2	1.3
n-Heptadecanoic	0.3	0.3	0.3
Palmitolinoleic	0.9	1.1	0.9
n-Hexadecadienoic	tr	0.5	0.3
Stearic	2.4	0.7	0.9
n-Hexadecatrienoic	1.3	2.7	3.5
Oleic	6.3	9.9	8.2
n-Nonadecanoic	0.5	0.2	0.4
Linoleic	6.8	6.1	7.7
Arachidic	0.8	0.4	0.4
γ-Linolenic	0.3	0.6	0.7
α-Linolenic	12.7	21.0	22.4
n-Eicosatrienoic	0.8	0.7	0.7
Unsaturated C_{20}	1.7	1.6	1.2
Arachidonic	1.1	3.3	4.0
Unsaturated C_{22}	tr	0.3	0.3
Unsaturated C_{20}	0.2	1.1	1.3
Lignoceric	6.3	3.7	5.4
Unknown	0.2	0.6	0.3

*See footnote to Table 1.

increase in unsaturated fatty acids occurs: their percentage increases from 36.1 in sample A (summer) to 52.3 in sample B (beginning of autumn), remaining unchanged (54%) in sample C (November). At the same time a gradual increase both of C_{18} (from 12.7 to 39.2%) and C_{20} fatty acids (from 4.1 to 7.6%) occurs accompanied by a decrease in C_{16} acids (from 57.6 to 44.7%). With a lowering of temperature lignoceric acid undergoes a decrease much less marked than palmitic acid, whereas among the unsaturated acids the increases more evident are those for ω-hexadecatrienoic acid amongst the C_{16} components, for oleic and α-linolenic acid among the C_{18} acids and, for polyunsaturated, amongst the C_{20} acids.

As shown in Table 2, two hexadecadienoic acids are present. To the first acid, indicated as palmitolinoleic, is tentatively assigned a 9,12-dienic structure on the basis of its ECL value. It should be noticed that, among the hexadecadienoic acids, only the isomer with double bonds in the 7,10 position was previously reported in other ferns [8].

The data of Table 3, relative to the unsaponifiable composition of *Azolla caroliniana*, shows that the hydrocarbon content rises continuously from summer to autumn. It is evident that the increase in the unsaponifiable content of *Azolla* lipids observed in sample C can be ascribed almost exclusively to the hydrocarbon fraction. The alcohol fraction has been studied by GC on both SE30 and OV 17. Peaks 1–12 are triterpenic alcohols (Table 4). In the same table the quantitative composition of the triterpenic components is reported. With the exception of peak 12 the major components were identified. The relative amounts of the main components of this fraction varied widely, particularly in sample B, when a marked increase in cycloartenol, accompanied by a decrease in 24-methylencycloartanol, occurred. Component 12 is always present in high relative percentages (19.5–30.8).

The composition of the sterol fraction (Table 5) does not vary. Contrary to what is found in algae, and particularly for cyanobacteria, typical sterols of higher plants are present, sitosterol being the main component.

EXPERIMENTAL

Azolla caroliniana Willd., obtained from the Botanical Garden of Florence, was mass cultured in outdoor conditions for seven months (from May to November). The ferns were grown in 25 m^2 surface ponds on a two-fifths Hoagland type nutrient soln [9]. The freeze-dried ferns were separately ground in a mortar with sand and extracted for 24 hr with MeOH–$CHCl_3$ (1:2) in a Paquot apparatus [10]. The solvent was removed by a rotary evaporator until a constant wt was obtained.

Total lipids were saponified with 1 N KOH in MeOH by refluxing for 3 hr. After addition of 2 vols of H_2O, the unsaponifiables were extracted with Et_2O. The aq. soap solns were acidified with 25% H_2SO_4 and the free fatty acids extracted with Et_2O. The extracts, washed with H_2O, were dried (Na_2SO_4) and the solvent removed by rotary evaporation. The free fatty acids were methylated with CH_2N_2–Et_2O at room temp. The corresponding Me esters were analysed by GC on a 2 m × 3 mm (id) glass column packed with 15% DEGS. Oven, injector and

Table 3. Unsaponifiable composition of the lipids of *Azolla caroliniana*, obtained by TLC densitometry (% of total unsaponifiable). Data in brackets are expressed in g/100 g dry matter

TLC band	R_f	Components	Sample* A	B	C
1	1.0–0.86	Hydrocarbons	21.9 (0.24)	31.7 (0.38)	48.3 (1.06)
2	0.80–0.40		16.2	27.1	12.2
3	0.33	Alcohols	22.9 (0.25)	17.6 (0.21)	8.3 (0.18)
4	0.25		3.3	3.3	2.5
5	0.19	Sterols	14.8 (0.16)	9.6 (0.11)	11.3 (0.25)
6	0.15–0.04		12.8	4.5	9.4
7	Start		8.1	6.2	8.0

*See footnote to Table 1.

Table 4. Triterpenic alcohol composition of lipids from *Azolla caroliniana* (% of alcohol fraction)

Peak	RRT* (SE30)	RRT* (OV17)	Components	Sample† A	B	C
1	0.88	—		tr	tr	0.3
2	0.94	—		0.9	0.8	0.8
3	1.00	1.00		tr	tr	tr
4	1.06	1.02	Cycloartanol	10.2	10.4	8.7
5	1.15	1.23	Cycloartenol	27.1	42.8	25.7
6	1.23	—		2.1	1.1	3.8
7	1.32	1.38	24-Methylene-cycloartanol	32.8	11.6	22.7
8	1.41	—		tr	1.2	0.7
9	1.47	2.05		tr	2.8	2.2
10	1.63	—		—	tr	tr
11	1.76	2.56		3.6	9.8	4.3
12	1.88	2.47		23.3	19.5	30.8

*Relative to β-sitosterol.
†See footnote to Table 1.

Table 5. Sterol composition of lipids of *Azolla caroliniana* biomass (% of sterol fraction)

Components	Sample* A	B	C
Cholesterol	0.7	1.1	0.8
Campesterol	10.4	10.6	14.0
Stigmasterol	0.4	0.6	0.3
β-Sitosterol	84.8	83.5	82.5
Δ^5-Avenasterol	2.6	2.9	2.4
Δ^7-Stigmastenol	1.1	1.3	—

*See footnote to Table 1.

detector (FID) isothermal temps were 190°, 220° and 220°, respectively.

Fractions obtained by $AgNO_3$ TLC [11] were analysed by GC using the experimental conditions described above.

The unsaponifiable extracts were washed with H_2O and dried (Na_2SO_4). The solvent was removed by rotary evaporation to constant wt. The unsaponifiables were then fractionated by prep. TLC. About 30 mg of each unsaponifiable (as a 10% soln in $CHCl_3$) was applied as a continuous band on the plates. Development was carried out with hexane–Et_2O (1.5:1). Bands were visualized with a 0.2% soln of 2,7-dichlorofluorescein sodium in 95% EtOH and observed under UV light (254 nm). Bands were scraped off and the adsorbent extracted with $CHCl_3$. The $CHCl_3$ soln of the recovered components was evaporated under N_2 and the residue silanized by treatment with pyridine–hexamethyldisilazane–trimethylchlorosilane (10:2:1). Silanized compounds were analysed by GC using a 3 m × 2 mm (id) glass column packed with 1.5% OV 17 and with a 3 m × 3 mm (id) glass column packed with 3% SE 30. The oven, injector and detector temps. were 270°, 280° and 280°, respectively.

For the densitometric determinations of the unsaponifiable compositions, TLC plates were sprayed with a satd soln of $K_2Cr_2O_7$ in 80% H_2SO_4 and heated in an oven for 25 min at 130°.

Acknowledgement—This work was supported by an Italian C.N.R. grant.

REFERENCES

1. Florenzano, G. (1983–84) *Ann. Accad. Agricol. Torino* **126**, 1.
2. Margheri, M. C., Materassi, R., Balloni, W. and Paoletti, C. (1979) *Agric. Ital.* **108**, 199.
3. Florenzano, G., Balloni, W., Materassi, R. and Torzillo, G. (1980) *Agric. Ital.* **109**, 283.
4. Margheri, M. C., Tomaselli, L. and Filippi, C. (1982). Workshop on practical application of *Azolla* for rice production, Mayagnez, Puerto Rico.
5. Favilli, F., Materassi, R., Balloni, W. and Florenzano, G. (1983) *Trans. VIIIth Int. Symp. 'Humus et Planta'*, Prague.
6. Vincenzini, M., Margheri, M. C. and Sili, C. (1985) *Plant Soil* **86**, 57.
7. Schlenk, H. and Gellerman, J. L. (1965) *J. Am. Oil Chem. Soc.* **42**, 504.
8. Nichols, B. W. and James, A. T. (1968) in *Progress in Phytochemistry* (Reinhold, L. and Liwschitz, eds.) Vol. I, p. 11. Wiley-Interscience, London.
9. Peters, G. A. and Mayne, B. C. (1974) *Plant Physiol.* **53**, 813.
10. Pham Quang, L., Laur, M. H. and Paquot, C. (1970) *Oléagineux* **25**, 223.
11. Pallotta, U. and Matarese, L. (1963) *Riv Ital. Sostanze Grasse* **40**, 579.