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Occurrence of diacylglyceryltrimethylhomoserines and major phospholipids in some plants

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Abstract

Over 40 higher plant species were examined for the contents of total lipids, phospholipids, diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) by using micro-HPTLC. The results showed a wider range of plants containing betaine lipids. So, DGTS was found in some higher plant species, not studied earlier, belonging to Equisetophyta, Polypodiophyta; the lipid composition of many other species from Spermatophyta was also studied. It was demonstrated that more primitive plant species contained, as a rule, the betaine lipid DGTS. The quantitative data for the distribution of the main phospholipid classes PC, PE, and PG in various plant species and their tissues are given in this paper. © 2000 Published by Elsevier Science Ltd.

Keywords: Plants; Ferns; DGTS; Lipids; Phospholipids; Occurence

1. Introduction

Presently, three basic types of betaine lipids of significant quantitative occurrence among polar lipid classes are known in plant kingdom; they are DGTS (diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine), DGTA (diacylglycerohydroxymethyl-*N*, *N*, *N*-trimethyl-β-alanine), and DGCC (diacylglycerylcarboxy-*N*-hydroxymethylcholine) (Dembitsky, 1996; Kato et al., 1996; Sato, 1992).

One of the main betaine lipids, DGTS, has been found in marine, fresh water (Dembitsky and Rozentsvet, 1989; Dembitsky et al., 1993b; Eichenberger, 1993) and brackish (Dembitsky and Rozentsvet, 1996; Jones and Harwood, 1992) green algae, as well as in bryophyta (Dembitsky, 1992; Dembitsky et al., 1993b; Bychek, 1994; Künzler and Eichenberger, 1997), lichens (Dembitsky, 1992; Dembitsky et al., 1993a), ferns (Sato, 1992; Sato and Furuya, 1984b; Dembitsky and Rezanka, 1995), and fungi (Dembitsky, 1996; Künzler and Eichenberger, 1997; Vaskovsky et al., 1991, 1998).

The questions relating to its subcellular localization and possible role in the lipid metabolism still remain unanswered, despite the fairly active development of investigations in this field. Since a DGTS molecule has a common structural fragment with phosphatidylcholine (PC) and many DGTS-producing organisms have a low or zero level of PC (Eichenberger, 1993; Kato et al., 1995), it can be supposed that these two lipid classes are interchangeable within the membrane structure. Moreover, in spite of their wide occurrence, betaine lipids are not found in all photosynthesizing plants (Dembitsky, 1996; Künzler and Eichenberger, 1997; Makewicz et al., 1997). As a rule, they are present in either lower plants or higher spore-producing ones. However, even these groups show a number of exceptions. Thus, not all green algae synthesize DGTS. Many examined fungal species do not contain DGTS (Vaskovsky et al., 1998). The latter is not present in one of the most ancient division of plants, Psilotophyta (Künzler and Eichenberger, 1997).

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Some fairly detailed studies on ferns (Künzler and Eichenberger, 1997; Sato and Furuya, 1984a, 1984b), mosses (Künzler and Eichenberger, 1997; Vaskovsky et al., 1991, 1998; Kato et al., 1995; Makewicz et al., 1997) have demonstrated that the existing taxonomic groups of these plants based on the presence or absence of DGTS can be either homogeneous, where all members of a taxon on the order or family level are DGTS-positive or DGTS-negative, or heterogeneous, comprising both DGTS-positive and DGTS-negative species (Dembitsky, 1996; Künzler and Eichenberger, 1997). However, there is an obvious lack of data, on either closely-related or systematically far apart species, to make the quantitative evaluation of DGTS possible.

The preliminary data on the qualitative distribution of betaine lipids in various plant and fungal groups are presently quite abundant (Dembitsky, 1996; Dembitsky and Rozentsvet, 1989, 1996; Vaskovsky et al., 1996, 1998). However, of major interest is the quantitative evaluation of DGTS and main phospholipids from various plant groups. Such data could bring the numerous preliminary studies to a new and higher level of chemotaxonomical and biochemical interpretation of the occurrence and composition of betaine lipids in the kingdom of plants and fungi.

The present study describes our continued investigation of plant betaine lipids.

2. Results and discussion

All presently known DGTS-producing plants are cryptogamic (Dembitsky, 1996; Sato, 1992; Sato and Furuya, 1984a, 1984b; Künzler and Eichenberger, 1997). In the frame of the present study we have attempted to widen the groups' boundaries of the plants already known as DGTS-positive. To do this, all the available plant species belonging to one and the same group and naturally growing in one and the same environment were selected. Thus, 13 fern and 6 horse-tail species were collected from the territory of the Zhiguli State Reserve. Also two other divisions of Sporophyta (Selaginellales and Psilotales) and two classes from the division Gymnospermae (Araucariales, Pinales, Cupressales, and Podocarpales), as well as a class of Cycadophyta (the order Cycadales), were examined.

Many authors who investigated DGTS-synthesizing organisms reported DGTS-positive and DGTS-negative species in principle only (Eichenberger, 1990, 1993; Künzler and Eichenberger, 1997; Vaskovsky et al., 1998) and showed just the qualitative characteristics of the distribution of DGTS and other betaine lipids. This approach seemed inadequate to us and we attempted a quantitative study of DGTS and major phospholipids.

For accurate separation of the polar lipids of interest, HPTLC was used (Belenky et al., 1984). DGTS, PC, PE (phosphatidylethanolamine), PG (phosphatidylglycerol), and other phospholipids were identified using the following specific spray reagents (Vaskovsky et al., 1975; Vaskovsky and Latyshev, 1975). To check the chemical structure, an additional procedure of DGTS isolation and its identification by IR and NMR spectral analysis was conducted. The IR spectrum of DGTS showed absorption bands 3000, 2960, 2920, 1390 cm^{-1} (CH_3– and CH_2– groups); 1740, 1450 cm^{-1} (CH_3–COO), 1580 cm^{-1} (COO–); 1370, 1030 cm^{-1} (C–N); 960 cm^{-1} (N–$(CH_3)_3$).

The PMR spectrum has signals at 0.87–0.89 ppm (CH_3), 1.23–1.34 ppm (–CH_2–); 1.62–1.82 ppm (–CH_2–C–O–); 2.02–2.08 ppm (CH_3–N); 3.72–3.75 ppm (CH_2–O); 4.29 ppm (CH–O–); 5.31 ppm (N–CH–COO). These characteristic bands correspond to the structure of DGTS as diacylglyceryl-*N*-*N*-*N*-trimethylhomoserine.

The data on DGTS amounts in the examined plants are reported in Table 1. The classification of plants was done as described (Flora UdSSR, 1934; Cherepanov, 1995). Obviously, only 20 species — out of 45 plants belonging to two divisions and seven classes — contained DGTS. Within the investigated Spermatophyta, there were no DGTS-containing species found, as described elsewhere (Jones and Harwood, 1992; Künzler and Eichenberger, 1997). The amount of this lipid in sporiferous plants varied widely from trace amounts to 49.4 mg/g lipid. In perfect agreement with previous studies (Künzler and Eichenberger, 1997), the species *Psilotum triquetrum* belonging to one of the most ancient sporiferous plants and occupying an isolated position among those presently existing, was not found to possess DGTS.

Lycopodium plants (Lycopodiophyta) were represented by the order Selaginellales. The analysis of three species of Selaginellaceae revealed rather a wide range of DGTS occurrence: 8.7 mg/g (*Selaginella kraussiana*) to 49.4 mg/g (*S. voqelii*). Since all the sample plants were collected at one time and from one place (the hothouse of Moscow Botanical Gardens), and of one and the same growth phase, the difference in DGTS composition can be related only to the species' distinctions.

Presently existing Equisetum plants were represented by one order (Equisetales) and one family (Equisetaceae). Six species from this systematic group revealed a remarkable variation of DGTS amounts: from traces in *Equisetum hyemale* to 21.1 mg/g lipid in *E. arvense*; it should be noted that the plants *E. arvense* (21.1 mg), *E. pratense* (8.8

Table 1
Distribution of the total lipids (TL, mg/g wet wt), total phospholipids (TPL, mg/g lipids), diacylglyceryltrimetylgomoserines (DGTS, mg/g lipids), phosphatidylcholin (PC)[a], phosphatidyletanolamine (PE)[a], and phosphatidylglycerol (PG)[a] in the plant kingdom

Division, SUBDIVISION, Class, Subclass, Order, Species	TL	TPL	PC	PE	PG	DGTS	Site and time of collection
Embryophyta							
SPOROPHYTA							
Psilotophyta							
Psilotopsida							
Psilotales							
Psilotaceae							
Psilotum triquetrum	14.8	6.2 ± 0.1	26.1 ± 0.4	12.2 ± 0.0	11.7 ± 0.0	–	1[b]
Lycopodiophyta							
Isoetopsida							
Selaginellales							
Selaginellaceae							
Selaginella kraussiana	10.0	2.3 ± 0.1	33.2 ± 1.7	16.1 ± 0.9	17.6 ± 1.0	8.7 ± 0.4	1[b]
Selaginella apus sp.	19.5	7.2 ± 1.3	34.7 ± 1.3	16.7 ± 0.7	13.2 ± 1.3	31.5 ± 1.5	1[b]
Selaginella vogelii	5.6	5.3 ± 0.2	18.3 ± 1.0	16.1 ± 0.7	13.0 ± 0.6	49.4 ± 2.9	1[b]
Equisetophyta							
Equisetopsida							
Equisetales							
Equisetum arvense	11.6	8.0 ± 0.2	23.2 ± 0.6	7.9 ± 0.6	16.7 ± 1.3	21.1 ± 2.1	2[c]
Equisetum pratense	16.9	8.2 ± 0.4	28.0 ± 1.3	5.3 ± 0.2	17.4 ± 0.4	8.8 ± 0.8	2[c]
Equisetum sylvaticum	13.8	5.1 ± 0.1	34.5 ± 0.9	10.8 ± 0.4	24.2 ± 0.8	16.3 ± 0.8	3[d]
Equisetum fluviatile	7.2	8.9 ± 0.4	26.9 ± 0.6	7.2 ± 0.2	23.5 ± 1.3	2.8 ± 0.1	3[d]
Equisetum trachyodon	9.3	9.4 ± 0.3	30.2 ± 0.6	11.6 ± 0.5	13.4 ± 0.1	8.6 ± 0.6	2[c]
Equisetum hyemale	8.0	10.0 ± 0.8	26.5 ± 0.4	10.2 ± 0.4	11.8 ± 0.2	Tr	2[c]
Polypodiophyta							
Polypodiopsida							
Polypodiales							
Onocleaceae							
Matteuccia struthiopteris	19.0	8.3 ± 0.3	27.3 ± 2.2	1.5 ± 0.1	17.4 ± 2.5	Tr	2[c]
Athyriaceae							
Athyrium filix-femina	12.3	6.9 ± 0.1	27.8 ± 0.9	8.5 ± 0.1	26.7 ± 2,6	11.8 ± 1.0	3[d]
Diplazium sibiricum	31.5	4.6 ± 0.1	32.0 ± 0.5	–	33.0 ± 0.7	6.4 ± 0.6	2[c]
Cystopteris fragilis	30.8	3.4 ± 0.1	31.2 ± 0.6	5.6 ± 0.2	15.0 ± 0.9	4.2 ± 0.4	2[c]
Gymnocarpium dryopteris	27.5	1.9 ± 0.1	25.0 ± 1.0	5.0 ± 0.7	25.0 ± 0.9	18.8 ± 0.4	3[d]
Gymnocarpium robertianum	20.1	4.2 ± 0.2	37.0 ± 0.4	–	13.0 ± 1.0	10.1 ± 0.6	2[c]
Dryopteris filix-mas	22.2	6.4 ± 1.4	40.1 ± 0.4	3.4 ± 0.5	15.1 ± 0.2	Tr	2[c]
Dryopteris carthusiana	23.1	8.0 ± 0.1	28.4 ± 1.0	5.1 ± 0.2	24.4 ± 0.2	4.2 ± 0.3	2[c]
Polystichum braunii	18.7	5.6 ± 0.3	32.5 ± 0.6	1.7 ± 0.1	21.7 ± 0.9	3.7 ± 0.3	2[c]
Thelypteris palustris	9.5	8.4 ± 0.1	27.0 ± 0.5	2.4 ± 0.1	11.3 ± 0.3	10.4 ± 1.0	2[c]
Aspleniaceae							
Asplenium ruta-muraria	28.0	4.1 + 0,2	36.6 ± 1.3	8.7 ± 0.8	13.9 ± 0.3	14.3 ± 1.4	2[c]
Asplenium trichomanes	22.0	8.3 ± 0.1	37.7 ± 0.4	5.5 ± 0.2	14.2 ± 0.1	12.6 ± 0.8	

(continued on next page)

Table 1 (*continued*)

Division, SUBDIVISION, Class, Subclass, Order, Species	TL	TPL	PC	PE	PG	DGTS	Site and time of collection
Hypolepidaceae							
Pteridium aquilinum	27.0	7.1 ± 0.3	26.4 ± 1.0	17.6 ± 0.3	20.8 ± 0.9	13.7 ± 1.1	2[c]
Salviniadae							
Salviniales							
Salviniaceae							
Salvinia natans	7.6	7.6 ± 0.2	38.9 ± 1.7	13.9 ± 0.6	13.9 ± 0.6	33.2 ± 1.6	2[c]
Marrattiopsida							
Marrattiales							
Marratiaceae							
Angiopteris evecta	14.4	6.1 ± 0.2	6.6 ± 0.9	2.8 ± 0.2	7.6 ± 0.2	6.9 ± 0.6	1[b]
SPERMATOPHYTA							
Gymnospermae							
Pinophyta							
Pinopsida							
Pinales							
Araucariaceae							
Araucaria angustifolia	28.0	2.3 ± 0.1	21.7 ± 1.7	10.1 ± 0.5	4.2 ± 0.3	–	1[b]
Araucaria columnaris	11.6	2.6 ± 0.1	10.5 ± 0.0	5.5 ± 0.0	3.3 ± 0.1	–	1[b]
Cupressaceae							
Cupressus sempervirens	33.4	2.8 ± 0.2	14.4 ± 0.1	4.4 ± 0.2	11.8 ± 0.6	–	1[b]
Cupressus lusitanica	24.8	3.7 ± 0.1	15.7 ± 0.5	4.8 ± 0.2	10.9 ± 0.3	–	
Cupressus pigmaea	30.0	3.2 ± 0.2	18.1 ± 0.9	2.8 ± 0.1	14.1 ± 0.2	–	1[b]
Cupressus glabra	41.6	2.4 ± 0.1	25.4 ± 0.3	6.9 ± 0.4	16.2 ± 1.4	–	1[b]
Juniperus sabina	93.6	1.0 ± 0.1	25.0 ± 1.5	5.0 ± 0.4	15.0 ± 0.4	–	3[d]
Pinaceae							
Pinus pinea	21.6	4.2 ± 0.1	17.3 ± 0.8	6.6 ± 0.3	8.6 ± 0.4	–	1[b]
Pinus halepensis	18.2	4.4 ± 0.2	9.3 ± 0.4	6.8 ± 0.3	4.5 ± 0.3	–	1[b]
Pinus densiflora	19.6	3.7 ± 0.3	11.5 ± 0.3	5.2 ± 0.1	8.2 ± 0.4	–	1[b]
Pinus yunnanensis	14.6	4.3 ± 0.1	19.7 ± 1.1	7.0 ± 0.4	8.1 ± 0.2	–	1[b]
Pinus sylvestris	88.9	1.0 ± 0.1	11.9 ± 0.2	2.3 ± 0.1	3.5 ± 0.1	–	3[d]
Podocarpaceae							
Podocarpus macrophyllus	15.8	4.4 ± 0.2	17.6 ± 0.7	7.4 ± 0.4	9.3 ± 0.6	–	1[b]
Podocarpus neriifolius	16.6	2.3 ± 0.1	12.4 ± 0.8	1.7 ± 0.1	8.0 ± 0.6	–	1[b]
Podocarpus acutifolius	38.9	2.2 ± 0.1	14.5 ± 0.5	11.6 ± 0.3	7.2 ± 0.6	–	1[b]
Podocarpus falcatus	26.5	2.7 ± 0.2	22.5 ± 1.5	8.2 ± 0.6	11.6 ± 0.3	–	1[b]
Podocarpus chinensis	23.8	2.7 ± 0.2	20.8 ± 0.8	9.4 ± 0.5	12.4 ± 0.3	–	1[b]
Cycadopsida							
Cycadaceae							
Encephalartos gilldebrandtii	31.2	2.1 ± 0.4	13.9 ± 0.2	5.0 ± 0.01	5.1 ± 0.4	–	1[b]
Encephalartos horridus	26.1	1.7 ± 0.1	24.7 ± 0.4	6.4 ± 0.7	20.4 ± 1.2	–	1[b]

[a] % Total phospholipids.
[b] 1: Moscow Botanical Garden, October 1998.
[c] 2: Zhiguli State Reserve, July 1997, 1998.
[d] 3: Zhiguli State Reserve, September, 1998.

mg), *E. trachyodon* (8.6 mg), and *E. hyemale* (traces) were collected at one and the same time.

The greatest number of species was from the division Polypodiophyta. They included 13 species of ferns growing in the natural environment of the reserve and one of the most ancient fern species (*Angiopteris evecta*) grown in the hothouse. The maximum DGTS was found in a water fern *Salvinia natans*, 33.2 mg/g lipid. Two species, *Matteuccia struthiopteris* and *Dryopteris filix-mas*, showed small amounts of DGTS which was quite unexpected for us. It is certainly difficult to determine the degree of relationship between individual plant groups on the basis of DGTS amounts since the number of species in each group is not high enough; however, some particularities can be noted: the amount of DGTS in the species from the family Athyriaceae collected during one and the same period was somewhat higher (12.6–14.8 mg/g) than in Dryopteridaceae where it showed significant variation from trace amounts (*D. filix-mas*) to 10.4 mg/g (*Thelypteris palustris*). It is hard to correlate the amounts of DGTS and the systematic position as the plant classification within this division is viewed differently in the (present day) literature.

Our quantitative examination of DGTS and phospholipids revealed other somewhat unexpected results. As seen from Table 1, all plant species possess PC. Moreover, it dominates the phospholipids in the majority of the examined species, contrary to PE the content of which appeared to be zero in certain species. The total amount of three phospholipids, PC, PE, and PG, in DGTS-containing species reached 50% or higher from total all phosphorus-containing lipids, thus allowing us to regard them as major phospholipids (MPL). The only exceptions were *A. evecta* (17.0%), *T. palustris* (40.8%), and *M. struthiopteris* (46.2%).

The correlation between MPLs has some interesting particularities as well. The content of PC in Equisetophyta (23.2–30.2% of TP) was lower than in Lycopodiophyta and varied within a narrower range while the content of PE (5.3–15.9% of TP) was lower than PG (13.4–24.2% of TP). The content of PC in ferns (25.0–40.1% of TP) was much higher than those of PE and PG. PE amounted to 1.5–17.6% being zero in two species (*Diplazium sibiricum* and *Gymnocarpium robertianum*), while DGTS in these species amounted to 6.4 and 10.1 mg/g, respectively.

As mentioned above, investigating DGTS-containing plants we checked a number of species belonging to Gymnospermae. Similarly to the sporiferous samples, the amounts of PC, PE, and PG were determined. It was revealed that the total of these lipids amounted to less than 50% of all total phospholipids which were evaluated relative to the total mineralized phosphorus (TPL). The examined plants appeared to possess certain similarities with regard to phospholipid contents. Thus, PC dominates the PC, PE, and PG group practically in all species studied. PG is second (3.3–20.4%) and PE third (2.8–11.6%) to PC in their contribution to the phospholipid content. The variation appears to be fairly wide when viewed in the frame of all species examined. However, the lower the level of the classification scale, the narrower are the variation limits of the lipid classes, and the more similarities can be revealed in the plants belonging to a given taxon. For example, Cupressaceae possess 14.4–25.4% PC, 2.8–6.9% PE, and 10.9–16.2% PG. All the plants from this family were grown in the environment of the hothouse similar to tropical conditions, except *Juniperus sabina* found in its natural habitat of the reserve. As seen from Table 1, the distribution of MPLs in these plants remains the same and does not depend on the place where they were collected, while the amounts of total lipids and total phospholipids vary considerably. Total lipids in *J. sabina* amount to 93.6 mg/g wet weight (wt). This is much higher than the figures found for other species belonging to the same family (24.6–41.6 mg/g wet wt), although the total phospholipids are notably lower (1.0 mg/g lipids) compared to other species (2.4–3.7 mg/g lipids).

The same regularities are also characteristic of the family Pinaceae. Four species belonging to this family were collected in the hothouse: *Pinus pinea*, *P. halepensis*, *P. densiflora*, *P. yunnanensis*; their natural habitat is the tropical climatic zone. One species collected in the reserve, *P. sylvestris*, usually grows in the moderate climatic zones. The similarities of lipid distribution within this family are also quite demonstrative. The first four species contain 14.6–21.6 mg/g wet wt TL, 3.7–4.3 mg TP/g lipids, 9.3–19.7% PC, 2.3–7.0% PE, and 3.5–8.6% PG. *P. sylvestris* has a similar ratio of MPLs but its GL content is much higher than that of other relative species (88.9 mg/g wet wt vs. 18.2–21.6 mg/g) while its total phosphorus, and therefore its phospholipids' content, is lower than in other species.

The obtained results suggest a new aspect of the problem of DGTS calling for further research. Particularly, a more systematic approach to the research into a wider range of species from one group is required in order to reveal DGTS-positive species. The produced amount of DGTS correlates with the systematic plant position. Besides, this amount obviously, reflects different steps in onthogenesis.

Along with elucidation of metabolic aspects, cellular localization, chemical structure of fatty acids etc. to better understand the fundamental role of DGTS in a cell, the search of new DGTS-producing organisms remains both actual and interesting as it can provide more data on the degree of relationship between

species and their evolution, as well as enable to make certain chemotaxonomic conclusions.

3. Experimental

The plant samples were collected from two different habitats. The first group belonging to sporiferous plants and including Equisetophyta and Polypodiophyta were collected from the territory of The Zhiguly State Reserve during two field seasons of 1997 and 1998. The two species of gymnospermous plants *P. sylvestris* and *J. sabina* were from the same environment. The major part of sample plants belonging to the division Gymnospermae, including Araucariales, Cupressales, Pinales, and Podocarpales, as well as the sporiferous Selaginellales and Psilotales, were collected during one period of time in the hothouse of Moscow Botanical Gardens.

The samples of Polypodiales, Pinales, Cupressales, Araucariales, Podocarpales were individual stems with leaves, taken from whole plants. Selaginellales and Psilotales were collected as average biomass (usually 2–5 g) consisting of a number of similar plants about 4–6 cm high without roots. Equisetophyta were also collected as average biomass of some plants, in vegetation phase, without the root system.

The samples selected for examination were cut to pieces, put into the chloroform–methanol mixture (2:1) and transported to the laboratory. In the laboratory, the samples were macerated in a high-frequency homogenization unit and extracted according to the method by Bligh and Dyer (1959).

The analysis of polar lipids was carried out using 6 × 6 cm plates with a fixed layer of silica gel, and two-dimensional chromatography with two solvent systems: chloroform–methanol–benzene–ammonia (130:60:20:12, v/v) for dimension I; chloroform–methanol–benzene–acetone–acetic acid (140:60:20:10:8, v/v) for dimension II.

To identify polar lipids, specific spray reagents were used: molybdenum blue (Vaskovsky et al., 1975) and malachite green (Vaskovsky and Latyshev, 1975) for phosphorus-containing lipids; Dragendorff's reagent (Kates, 1975) for choline-containing lipids; 0.2% ninhydrin solution in acetone (Kates, 1975) for amine-containing lipids; anthrone reagent (Practicum of biochemistry, 1989) for glycolipids.

IR spectra were obtained using an IR spectrophotometer "Specord M82" (Carl Zeiss, Jena, Germany) in a fine layer using KBr. PMR spectra were obtained using a spectrometer "Bruker AM-300" (^{1}H 300 MHz; ^{13}C 7547 MHz) (Karlsruhe, Germany), with tetramethylsilane as the internal standard, and $CDCl_3$ as the solvent.

The quantitative analysis of phospholipids and DGTS was conducted as follows: the total lipid extracts were separated by two-dimensional chromatography; the spots were visualized by spraying them with 10% solution of H_2SO_4 in methanol followed by the determination of the phosphorus content in phosphorus-containing spots according to Vaskovsky and Latyshev (1975). The spectrometric determination of DGTS amounts was done as described previously (Dembitsky and Rozentsvet, 1989). The calibration curve was drawn based on the known DGTS concentrations: 1, 2, 5, 10, 15, 20 μg/g.

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