Characterization of γ-Linolenic Acid in *Ribes* Seed

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ABSTRACT

The total lipid content of fruit seeds of the *Ribes* family ranges by weight from 18.3% in gooseberries *(Ribes uva crispa)* to 30.5% in black currants *(Ribes nigrum)*. Isolation procedures and analytical methods (gas chromatography, mass spectrometry, high performance thin layer chromatography and stereospecific analysis) demonstrate that the oils from *Ribes* seeds contain up to 19% by weight of γ-linolenic acid (γ-LA, C18:3, n-6) in black currant oil. This last *Ribes* species thus constitutes one of the richest natural sources in γ-LA yet described. These oils appear promising for critically ill patients who seem unable to convert linoleic acid into subsequent EFA fractions.
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INTRODUCTION

γ-linolenic acid (γ-LA, C18:3 n-6) is known to play a crucial step in the generation of prostaglandin derivatives (1). Under normal physiological circumstances, γ-LA results from the hepatic bioconversion of linoleic acid (LA, C18:2 n-6), the major essential fatty acid (EFA) for humans. The transformation of LA to the more unsaturated γ-LA requires the activation of the liver Δ6-desaturase enzyme (2). The dietary requirements for LA are estimated around 2.7% of the total caloric intake in children (3) and around 3-5 g/day in adults (4). These EFA amounts usually are supplied with a well-balanced diet. The endogenous conversion of LA into γ-LA and subsequent compounds proceeds normally, explaining why biochemical or clinical signs of EFA deficit are extremely rare.

In contrast, it is known that fat-free parenteral diet very rapidly exhausts the endogenous EFA resources, leading to biochemical and clinical abnormalities (5-8). Moreover, a number of recent reports suggest that the normal transformation of LA into further EFA fractions may be depressed under several stressful conditions (9-11), most probably as a result of the Δ6-desaturase depression (2). Critically ill patients thus become at risk of developing EFA-deficient status, even in the case of appropriate LA delivery. We have, therefore, focused our attention towards new lipid sources which could be of clinical usefulness in situations characterized by the enzyme defect. We were successful in isolating in the seeds of fruits belonging to the *Ribes* family varying γ-LA concentrations, amounting to 19% in black currant oil (12).

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ISOLATION PROCEDURES

In black currant varieties from Austria, France, Germany, Sweden and Switzerland, the amount of γ-LA varied within relatively narrow limits. Principally 3 different sources are available for the oil extraction from black currant seeds. These are: [1] whole fruits, including possibly enzymatic treatment for obtaining the seeds; [2] industrial residues (press cakes) from juice or jam production, and [3] industrial residues from fermentation processes, which are products of [2].

According to the degree of purification of the raw material, their fat content may vary between 13 and 30%, the latter being the amount of fat in washed grains. The press cake is milled and extracted on a Soxhlet apparatus with hexane to yield an oil which contains a large amount of wax and dyestuff, 7-8%. After their removal by winterization and bleaching, the resulting oil was analyzed.

Gas Chromatography (GC) Analysis

GC analyses of fatty acid methyl esters (FAMES) (13,14) were performed on tailor-made high-resolution Carbowax 20 M capillary columns (15,16). For this purpose the triglycerides were transmethylated with sodium methoxide (17,18). Figure 1 shows a typical GC of FAMES of black currant oil with the sequence: palmitic, stearic, oleic, elaidic, linoleic, γ-linolenic, α-linolenic, stearidonic and very little arachidic and gadoleic acid. The range of composition, according to the origin of the seeds, is shown in the legend. Each of these peaks was identified by retention time in comparison with known standards, except for stearidonic acid. Utilization of other stationary phases, like a mixture of OV-17 and SE-30 (1:1), and SP 2340, gave the same results.

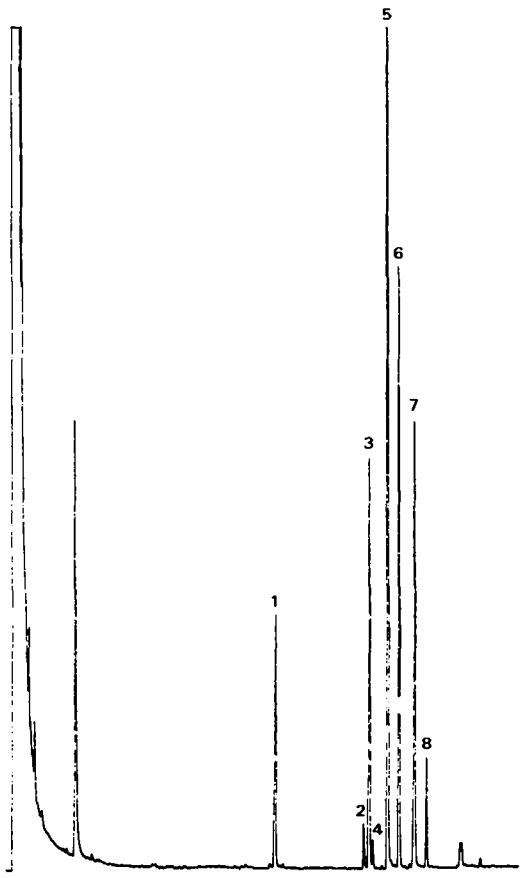
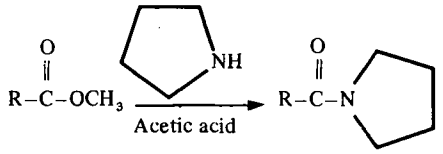


FIG. 1. GC of black currant oil FAMES. Conditions: Carlo Erba Mod. 4160 with high resolution Carbowax 20 M capillary column. On column injection, 80-230 C. For details, see references 15 and 16.

Peak number	FAMES	% of total lipid FAME
1	C16:0	6-7
2	C18:0	1-2
3	C18:1 N-9 *cis*	9-10
4	C18:1 N-9 *trans*	0.5
5	C18:2 N-6 all *cis*	47-49
6	C18:3 N-6 all *cis* (γ-linolenic acid)	15-19
7	C18:3 N-3 all *cis*	12-14
8	C18:4 N-3 all *cis* (stearidonic acid)	3-4
U	unknown (from solvent)	

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

Electron impact mass spectra of FAMES do not give structural information on double bond positions (19-24). To obtain these data FAMES or triglycerides may be converted into pyrrolidides (25).



The yield of this reaction under the described conditions is slightly over 90% and still some unreacted FAMES remain in the mixture. Our works demonstrated almost 100% conversion into the pyrrolidides when triglycerides were used as substrate instead of FAMES. Obviously, glycerol is a better leaving group than the methoxyl during substitution. A typical procedure was: 10 mg oil, 1 ml pyrrolidine and 0.1 ml acetic acid were stirred for 30 min at 100 C in a closed, round bottomed flask. Unreacted solvents were evaporated on a vacuum evaporator for about 30 min at 90 C bath temperature. The residue was taken up in heptane to give a 0.05% solution of which 1 μl was injected for GC/MS. However, GC-retention indices of the pyrrolidides are different from those of the methyl esters. The pyrrolidides of linoleic and γ-linolenic acid show very similar retention behavior on Carbowax columns and thus are very difficult to separate. This problem becomes even more pronounced in case of GC/MS, as peak broadening effects due to the interface result in reduced resolutions. On the other hand, α-linolenic pyrrolidide is well separated from the 2 n-6 acids 18:2 and 18:3, but shows the same resolution problem with stearidonic pyrrolidide 18:4 n-3. The basic separation principle of different compounds on Carbowax columns is related to their polarity. It appears that in the case of fatty acid pyrrolidides the polarity of the compounds is highly influenced by the positions of the double bonds, in particular those of $\Delta 6$ and $\Delta 9$, respectively, relative to the amide group, Figures 2a and 2b show the corresponding mass spectra of the pyrrolidides of γ-linolenic and stearidonic acids, respectively.

Fragmentation patterns of the different compounds show the exact positions of double bonds in each of the acids. The amide group obviously has a charge stabilization effect upon the fatty acid moiety (26-28) which results in more stable and characteristic fragments. According to the general rule, fragment intervals of 12 mass units which occur between the most intensive peaks of clusters of fragments containing n and n-1 carbon atoms of the acid moiety, indicate the double bond being located between carbons n and n+1. Accordingly, the EI-mass fragmentation of γ-linolenic pyrrolidide shows intervals of 12 mass units between carbons 5 and 6, 8 and 9 and 11 and 12, respectively

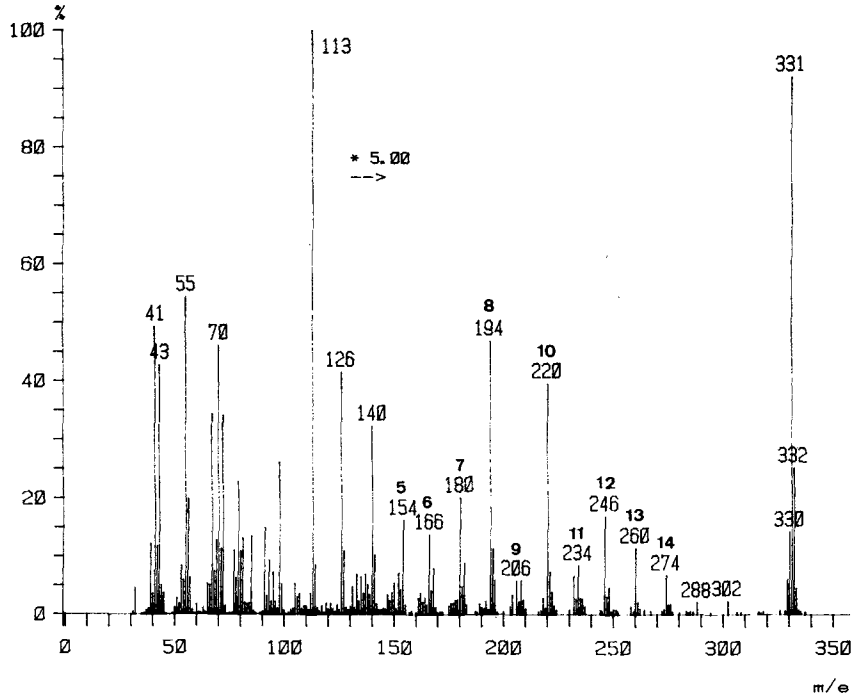


FIG. 2a. MS of N-octadec-6,9,12-trienoylpyrrolidide. Conditions: EI ionization 70 eV, 200 C (Kratos MS 30).

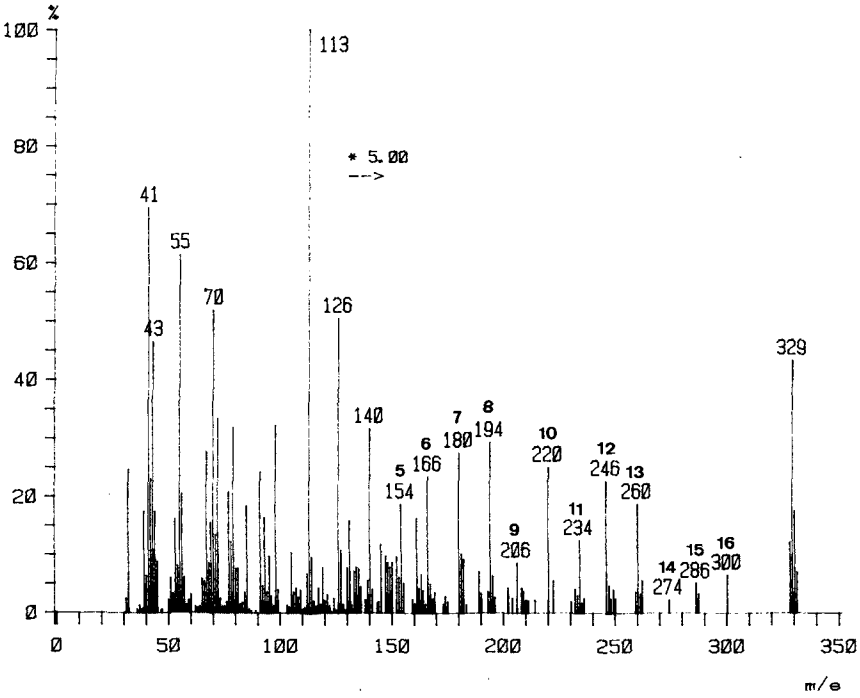


FIG. 2b. MS of N-Octadec-6,9,12,15-tetraenoylpyrrolidide. Same conditions as in Figure 2a.

(Fig. 2a). The presence of stearidonic acid C18:4, n-3 in black currant oil also could be proved by this method (Fig. 2b).

Preparative High Performance Thin Layer Chromatography (HPTLC)

As already mentioned above, GC separation of some of the fatty acid pyrrolidides was not sufficient for the production of well resolved peaks and clearly defined mass spectra. Therefore, a preparative preseparation of FAMES on thin layer plates had to be performed. Black currant oil was saponified and methylated with diazomethane in ether and a group separation according to the degree of unsaturation was achieved on reversed phase RP-18 as well as RP-12 HPTLC plates (A. Studer and H. Traitler, in preparation). Argentation chromatography which basically would lead to the same results was abandoned due to its inconvenience. Moreover, separation efficiencies are not better with the latter and quantification by visible scanning over the spots is practically impossible in the case of $AgNO_3$. Linoleic acid could be separated from γ- and α-linolenic acid, which migrated together, and stearidonic acid (18:4, n-3), the latter giving a single and very pure spot. Figure 3 shows a chromatogram scanned over a FAMES separation on RP-18 HPTLC plates. Conversion of the fractionated FAMES to the pyrrolidides gave ideal samples for further GC/MS analysis.

Analysis of Geometric Isomers

In order to obtain evidence on the stereoisomerism of the double bonds of the black currant oil fatty acids, an enzymatic analysis with lipoxygenase was performed (29-33). For this purpose 120 mg of oil were saponified with ethanolic potassium hydroxide solution; borate buffer and 0.5 N hydrochloric acid were added. After 20 min, an aliquot of this solution together with inactivated lipoxygenase was used for zero adjustment. Samples were measured with active lipoxygenase after a reaction time of 20 min. This method allows the determination of the total amount of *cis* double bonds in isolated pentadienoic structures and can be used for the quantification of *cis* double bonds in polyunsaturated fatty acids as well. For this purpose, black currant oil FAMES were submitted to an enzymatic analysis. Only those polyunsaturated fatty acids having *cis* double bonds in $\Delta 9$ and $\Delta 12$ positions are susceptible to lipoxygenase catalyzed oxydation to hydroperoxide (Fig. 4). This compound shows a UV absorption maximum of 234 nm and thus can ideally be used for the quantitative determination of most polyunsaturated fatty acids also in complex mixtures.

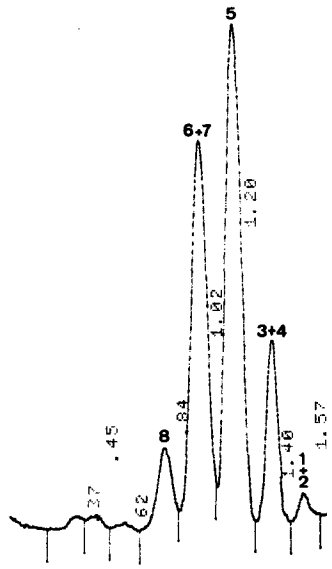


FIG. 3. HPTLC densitogram of black currant oil FAMES. Conditions: Nanoplate RP 18 (Merck) with solvents methanol/acetonitrile 1:1 dipping into 5% phosphomolybdic acid in acetone, charring at 150 C, 5 min. Scanning: Camag scanner, 546 nm, scanning speed 0.5 mm/sec. slit width 5 mm. Integration: Spectra Physics 4100, chart speed 4 cm/min, attenuation 128. (For peak numbers, see Fig. 1.) Vertical figures indicate retention times as printed out by the integrator.

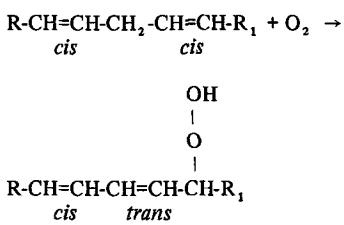


FIG. 4. Schematic pathway of lipoxygenase reaction.

The total amount of polyenic *cis-cis* compounds in black currant oil as determined gas chromatographically was 81.9%. Application of the enzymatic method, including the calibration with pure linoleic acid, gave a value of 83.7% polyunsaturated fatty acids. The conclusion can be drawn from these results that all polyunsaturated fatty acids in black currant oil also have all-*cis* double bonds.

DISCUSSION

The present study unequivocally demon-

TABLE 1

Linoleic Acid, γ-Linolenic Acid, Dihomo-γ-Linolenic Acid and Arachidonic Acid in Some Natural Products

Natural sources	Total lipid content	EFA fractions			
	g/100 ml (milks) g/100 g (seeds)	LA[b]	γ-LA[c]	DHγLA[d]	AA[e]
		(% of the total lipid content)			
Human colostrum[a]	2.5-3.5	7.82	0.34	0.49	0.71
Human mature milk[a]	3.5-4.5	10.75	0.35	0.31	0.41
Hops seeds (Humulus lupulus)	7.0	52.8	3-4	0	0
Hemp seeds (Cannabis sativa)	38.0	56.4	3-6	0	0
Red Currant Seeds (Ribes rubrum)	25.2	41.5	4-6	0	0
Evening Primrose Seeds (Oenothera biennis)	17	71.5	7-9	0	0
Gooseberry seeds (Ribes uva crispa)	18.3	40.0	10-12	0	0
Black Currant Seeds (Ribes nigrum)	30.5	48.5	15-19	0	0

[a]Values from Gibson and Kneebone, 1981 (ref. 36).

[b]LA = linoleic acid, C18:2 n-6.

[c]γ-LA = γ-linolenic acid, C18:3 n-6.

[d]DHγLA = dihomo-γ-linolenic acid, C20:3 n-6.

[e]AA = arachidonic acid, C20:4 n-6.

strates the presence of γ-LA in several fruit seeds belonging to the *Ribes* family, with the richest concentration reaching up to 19% of the total lipid content in seeds of black currant oil. This seed oil also contains 13.5% of α-linoleic acid (α-LA, C18:3 n-3) and 3.5% of the unusual stearidonic acid (SA, C18:4 Δ6,9,12,15 n-3) fraction, setting black currant oil in a very original position as regards EFA sources. Both α-LA and SA belong to the n-3 PUFA series leading to the formation of n-3 eicosapentaenoic acid (EPA, C20:5 n-3) and of docosahexaenoic acid (DHA, C22:6 n-3), a bioconversion requiring the same desaturases and elongases as those involved in the generation of n-6 and n-9 compounds. The n-3, n-6 and n-9 anabolic pathways are, therefore, in competition for the elaboration of their end-products. A recent patent (12) covers the fruit seeds of the *Ribes* family as purveyors of the physiologically active n-6 EFA fractions.

Healthy newborns submitted to breast-feeding are not at risk of developing signs of EFA deficit. Depending on maternal diet and nutritional status, large fluctuations in the LA content of human milk are described (34). Under normal circumstances, however, the LA concentration of human colostrum and milk lies within relatively narrow limits oscillating from 7% to 11% by weight of the total lipid content (35, 36). This means that LA intake by breast-fed children is significantly higher than that supplied by a well-balanced diet during any later period of life. The additional presence of γ-LA, dihomo-γ-linolenic acid (DHγLA, C20:3 n-6) and AA in human colostrum and milk (35,36) points to the uniqueness of breast-feeding and further suggests that EFA compounds fulfill special requirements in the growing child during the first months of life. Table 1 compares some EFA characteristics of human milk, 3 already described vegetable oils and 3 lipid fractions extracted from *Ribes* seeds.

Body EFA resources are very low at birth (1) so that fat-free enteral or parenteral diet causes biochemical and clinical abnormalities both in premature infants (5) and in healthy newborns (6,7). Biochemical signs of EFA-deprivation arise as early as one week after the onset of fat restriction (7,37) whereas clinical signs, such as dermatitis, usually develop after 3 weeks (38). Even adult patients with appropriate reserves of linoleate in adipose tissue triglycerides may exhibit early biochemical and clinical signs of EFA depletion (8,39). This situation typically is delineated by a gradual decrease in the long chain n-6 derivatives, contrasting with a progressive increase in endogenous FA substitutes for carbon chain elongation and desaturation. Theoretically, administration of lipid emulsions based on soybean oil or safflower oil would suffice to prevent biochemical and clinical symptoms of EFA deficiency, since these 2 vegetable products contain

54% and 77.5% LA, respectively. An increasing number of recent studies, however, demonstrate that use of emulsions containing LA may not solve the problem of EFA deficiency in total parenteral nutrition (TPN), since the blood and tissues of those patients contain significantly higher LA but lower arachidonate levels, a metabolic profile strongly suggestive of depressed bioconversion (6,7,8), entailing the accumulation of LA. Moreover, the major PG_1 and PG_2 urinary metabolites significantly decline during the course of TPN (9) to reach nadir values reminiscent of EFA deficient status (40). The most likely explanation resides in alterations of the $\Delta 6$-desaturase activity which seems reset at novel functional thresholds favoring the generation of n-9 derivatives at the expense of n-6 compounds. The current consensus is that the $\Delta 6$-desaturation is the rate limiting step in linoleic acid metabolism (41,42) and that the enzymatic activity may be inhibited by a number of factors such as protein restriction (43), fasting, premedication and anesthesia (44), and several nutritional or hormonal alterations (2) that are involved in the stress reaction (45). These data suggest that currently available lipid emulsions, despite their helpful supply of energy, may well not optimally fulfill the true EFA requirements of critically ill patients who, therefore, appear as major candidates for the beneficial effects of γ-LA supplementation.

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