Technical

Fractionation of Blackcurrant Seed Oil

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Blackcurrant seed oil is known to be one of the richest natural sources of γ-linolenic (all *cis*-6,9,12-octadecatrienoic) acid, with values of up to 20% of this acid. These concentrations are sufficient for most applications of the oil, but some utilizations require higher concentrations of γ-linolenic acid. Blackcurrant seed oil also contains up to 14% α-linolenic (all *cis*-9,12,15-octadecatrienoic) acid. Different fractionation techniques have been evaluated to separate γ-linolenic acid specifically from the other fatty acids present in the oil and, in particular, from α-linolenic acid.

Distillation as well as fractionated crystallization at various temperatures did not give any reasonable results. Surprisingly enough, urea fractionation in methanol gives a specific separation of α- and γ-linolenic acid, whereas stearidonic (all *cis*-6,9,12,15-octadecatetraenoic) acid, which is present at around 3% in the blackcurrant seed oil, cannot be separated by urea fractionation.

Stearidonic acid, like γ-linolenic acid, has a double bond in the Δ6 position, which makes these two acids unique in this respect. This most probably explains their similar behavior toward urea-occlusion.

Further semi-industrial preparative HPLC separations allowed us to obtain fractions of 95% γ-linolenic acid.

Linoleic (all *cis*-9,12-octadecadienoic) acid and its homologues are known to have important biological activity as precursors of eicosanoids which regulate functions such as arterial pressure, contraction of smooth muscles, platelet aggregation, and contraction and dilation of vascular glands. It has been suggested that the etiology of certain diseases may derive from a relative deficiency, not of linoleic acid but of its homologues such as γ-linolenic (all *cis*-6,9,12-octadecatrienoic) acid (GLA), dihomo-γ-linolenic (all *cis*-8,11,14-eicosatrienoic) acid or arachidonic (all *cis*-5,8,11,14-eicosatetraneoic) acid. This could arise from an inadequate transformation of linoleic acid to γ-linolenic acid catalyzed by the liver Δ6 desaturase (1). A number of reports assume that this bioconversion is indeed depressed under several stressful conditions (2-4), most probably resulting from a Δ6 desaturase enzyme defect. In such cases an essential fatty acid deficient status may be avoided by direct intake of GLA. There are only few significant natural sources of GLA. It is found in triglycerides of, e.g., borage, evening primrose, hemp and hopseed oils.

During a recent study GLA was detected in a series of other seed oils of the Boraginaceae, Scrophulariaceae, Onagraceae and Saxifragaceae families (5). Furthermore, the presence of GLA was reported in several fruit seeds belonging to the Ribes family, the richest being up to 20% of the total lipid content in blackcurrant seed oil (6,7).

Table 1 lists the fatty acid compositions of the most important known GLA sources. One can see that borage seed oil and blackcurrant seed oil have the highest GLA contents, with values up to 21-25% for borage seed oil and 15-20% for blackcurrant seed oil. The GLA concentration in gooseberry, evening primrose and red currant seed oil is inferior, and for gooseberry oil is in the region of 10-12%, for evening primrose oil 7-10% and for red currant seed oil 4-5%. Contrary to borage oil and evening primrose oil, the seed oils of the Ribes family contain α-linolenic (all *cis*-9,12,15-octadecatrienoic) and stearidonic acid. The latter, which is also of biological interest as it is formed in the lipid metabolism from α-linolenic acid by Δ6 desaturase, is rarely found in nature. The above mentioned GLA concentrations are sufficient for most applications, but several uses, in particular pharmaceutical ones, require higher concentrations of GLA, sometimes up to 90% or more. In this paper a fractionation technique for enriching GLA from blackcurrant seed oil will be reported.

EXPERIMENTAL PROCEDURES

Preparation of fatty acids. One kg refined blackcurrant seed oil (BCO) was mixed with 2.13 kg of an alkaline solution composed of 47.75% water, 14.3% NaOH, 0.15% Na_2EDTA and 37.8% ethanol (95%) at 60 C for 30 min (all chemicals from E. Merck, Darmstadt, Federal Republic of Germany). After addition of 0.4 kg water the solution was acidified at a temperature of 30-40 C with one kg HCl 32% and treated with 1.7 kg hexane for 30 min. The upper hexane layer was then

TABLE 1

Fatty Acid Composition (in %) of Several γ-Linolenic Acid-Containing Seed Oils

Fatty acids	Borage	Blackcurrant	Gooseberry	Evening Primrose	Red currant
C16:0	9-11	6- 8	7- 8	5- 7	4- 5
C18:0	2- 4	1- 2	1	1- 2	1- 2
C18:1,Δ9	14-18	9-13	15-18	5-10	14-16
C18:2,Δ9,12	35-40	44-51	39-41	73-78	41-42
C18:3,Δ6,9,12 (γ)	21-25	15-20	10-12	7-10	4- 5
C18:3,Δ9,12,15 (α)	-	12-14	19-20	-	29-31
C18:4,Δ6,9,12,15	-	2- 4	4- 5	-	2- 4
Other	5-10	< 2	< 2	< 2	< 2

TABLE 2

Preparative Liquid Chromatography Conditions for Concentrating γ-Linolenic Acid

	Prep LC/system 500	Kiloprep 1010 system
Code	A	B
Eluent	90% methanol 10% water	90% methanol 10% water
Column	2 5.7- × 30-cm columns in series	1 20- × 60-cm column
Packing material	RP C-18	RP C-18
Flow rate	150 ml/min	2 l/min
Sample	10 ml of 20% fatty acids in eluent	100 g fatty acids in 600 ml eluent
Detector	Refractive index	Refractive index
Chamber pressure	15 bar	10 bar

separated and the solvent evaporated, yielding ca. 0.9 kg fatty acids.

Fractionation with urea. One kg fatty acids (FA), 3 kg urea (U) crystallized extra pure (E. Merck, Darmstadt, Federal Republic of Germany) and 6.3 kg methanol were heated with stirring to 65 C to obtain a clear solution which was slowly cooled to ca. 4 C with slight stirring and then stored at this temperature overnight for completing the crystallization of urea and urea complexes. After filteration under vacuum one kg of the liquid phase was mixed with 0.13 kg hexane, 0.11 kg HCl (32%) and 0.29 kg H_2O. The hexane phase was separated and the water phase was extracted a second time with hexane. The combined hexane phases were evaporated, yielding 10–14% of a crude GLA concentrate.

Analysis of the fatty acids. Analysis of the composition of the fatty acid concentrates was performed by capillary gas chromatography. The FA were methylated with diazomethane, (from 1-methyl-3-nitro-1-nitrosoguanidine, Aldrich Chemical Co., Milwaukee, Wisconsin) and the fatty acid methyl esters were quantitatively determined with a Carlo Erba gas chromatograph Series 4160 (Carlo Erba Strumentazione, Milan, Italy) equipped with a cold on-column injector and a flame ionization detector on a high resolution Carbowax 20M capillary column. The fatty acids were identified by retention time in comparison with known standards or by gas chromatography/mass spectrometry analysis (7).

Fractionation by chromatographic methods. Preparative high performance liquid chromatography (HPLC) was applied to the GLA concentrate obtained by urea fractionation for separation of GLA from stearidonic acid. Appropriate separation conditions were developed on C18 reversed phase high performance thin layer chromatography (HPTLC) and transferred to a g-scale liquid chromatograph Prep LC/System 500 (Waters Associates, Framingham, Massachusetts) equipped with two 5.7 × 30 cm columns packed with PrepPak® 500/C18 cartridges (Waters Associates, Framingham, Massachusetts), particle size 55–105 micron. Further scaling up of this separation was carried out with a Kiloprep KP 1010 liquid chromatograph (Millipore Corp., Bedford, Massachusetts) equipped with a 20 × 60 column filled with the same packing material. The conditions applied are summarized in Table 2. A three-component mobile phase (methanol:ethanol:water, 67.5:22.5:10) as described in (8) for Prep 500 separations was modified to a two-compound eluent (methanol:water, 90:10) which is more practical for large-scale operations and solvent recovery. Both instruments were equipped with refractive index detectors. The pressure drop over the columns was about 15 bar for the Prep 500 and about 10 bar for the Kiloprep KP 1010 system. The injected solutions contained two g of fatty acids for the Prep 500 and up to 100 g for the Kiloprep KP 1010 system. For the latter system the loading may still be increased. The total run-time was about 20 min allowing, under optimum conditions, three injections per hr corresponding to 7.2 kg in 24 hr when applying 100 g fatty acids at each injection.

RESULTS AND DISCUSSION

Enrichment in γ-linolenic acid by urea-fractionation. Blackcurrant seeds are available in large quantities in the form of pomace remaining as residue from the production of jams, jellies, juices or liquors. The oil can be obtained from these seeds, which are washed with alcohol to eliminate waxes and coloring agents, by pressing or extracting with hexane. Subsequent refining is carried out in the classical way, i.e., degumming, neutralization, bleaching and deodorization. As γ-

TABLE 3

Composition of Blackcurrant Seed Oil Fatty Acids Before and After Urea Fractionation

Fatty acids	Before fractionation (%)	After fractionation (%)
C16:0	6.4	-
C18:0	1.3	-
C18:1	10.3	-
C18:2	46.5	2.3
C18:3γ	18.2	79.6
C18:3α	13.6	2.6
C18:4	3.7	15.5

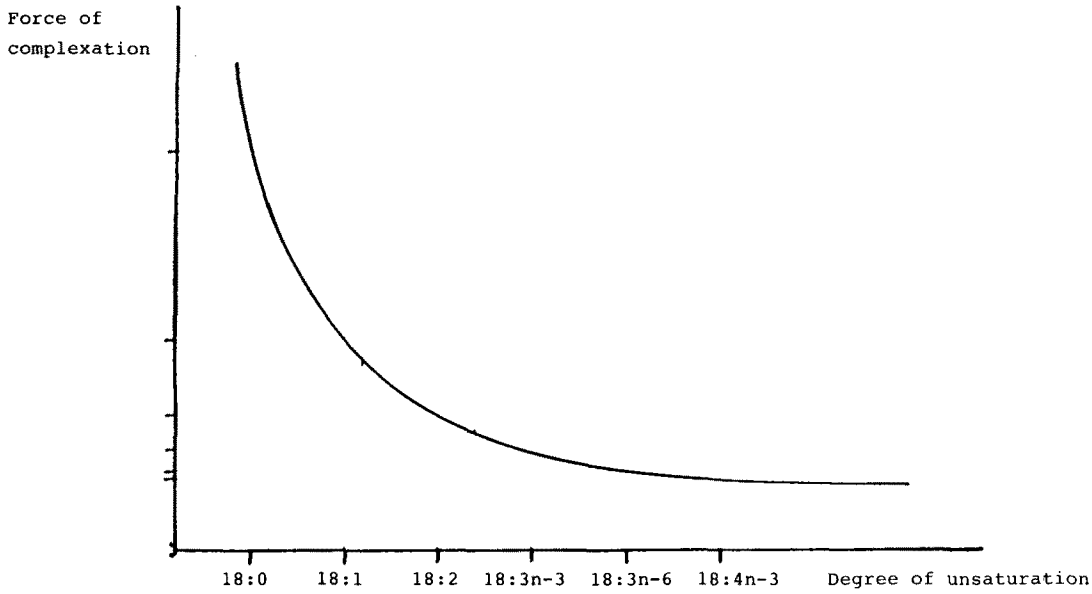

FIG. 1. Idealized scheme illustrating force of complexation of urea versus degree of unsaturation of a series of fatty acids.

linolenic acid cannot be concentrated by fractionation of BCO in its triglyceride form, it is necessary to transform it into fatty acids or esters. Different fractionation techniques were tested. Neither distillation nor fractional crystallization at various temperatures gave reasonable results. Preparative HPLC gave separations on reversed phase columns (RP-18 type) but α- and γ-linolenic acid which are present in blackcurrant seed oil in similar concentrations couldn't be separated by this technique. It could be shown that the urea fractionation method gave an excellent specific separation of α- and γ-linolenic acid (8). Urea forms crystalline inclusion compounds with fatty acids (9). Usually urea crystallizes in a tetragonal structure. An x-ray study showed that urea occlusion compounds consist of hexagonal crystals forming canals which are capable of occluding long-chain molecules (10). The formation of these inclusion compounds depends on the degree of unsaturation of the fatty acids; the more they are unsaturated the less they will be included in the urea crystals. This method was widely used for fatty acid fractionations, especially for analytical purposes. Among others it was also applied to evening primrose oil (11).

The fatty acids needed for the fractionation were produced by saponification as described under experimental procedures. For an industrial application it might be reasonable to produce the acids by fat splitting. The saponification could be started directly on milled or floculated blackcurrant seeds but preferably on the oil extracted with hexane. As well as urea, thiourea and dioxycholic acid form inclusion compounds, but only urea as the complexing agent enabled concentration of GLA. The result of such a fractionation is presented in Table 3, showing a concentration range of GLA from 18.2 to 79.6% and of stearidonic acid from 3.7 to 15.5% and a decrease of α-linolenic acid from 13.6 to 2.6%. The fatty acids were, for the most part, separated according to their degree of unsaturation. In this case the saturated, mono- and diunsaturated acids were separated from the more polyunsaturated acids. Unexpectedly, α- and γ-linolenic acids, which have the same degree of unsaturation, were successfully separated. On the other hand, this fractionation also enriched in stearidonic acid. By this method, GLA, an n-6 acid, and stearidonic acid, an n-3 acid, were cofractionated, leading to a product whose typical composition was ca. 80% GLA and 15% stearidonic acid. The similar behavior of these acids toward urea inclusion probably can be explained by the fact that both acids have a double bond in the Δ6 position, which is not the case for any of the other acids, making them unique in this respect.

Figure 1 illustrates, in an idealized form, a plot of the forces of complexation of urea versus fatty acids with different degrees of unsaturation. The complexation force of α-linolenic acid is stronger than that of GLA and stearidonic acid, which are in the same order. This corresponds to the experimental findings.

TABLE 4

Optimization of Urea Fractionation Conditions by Variation of Reaction Parameters

Fatty acids:urea ratio	1:2	1:3	1:4	1:5		
γ-Linolenic acid (%)	40.4	78.9	79.4	79.5		
Urea:methanol ratio	1:2.1	1:2.5	1:3			
γ-Linolenic acid (%)	78.9	74.7	64.9			
End temperature (°C)	+10	+8	+4	+2	+0	-15
γ-Linolenic acid (%)	69.1	72.4	76.5	79.0	79.3	79.0

Optimization of reaction conditions. The above-mentioned process was obtained by a detailed study of the reaction conditions. The results of the variation of some parameters are described in Table 4. The ratio of fatty acid/urea had to be 1:3 or higher. If not enough urea was present, GLA concentration in the final product decreased. The urea/methanol ratio had to be chosen in such a way as to obtain a clear solution at 65 C, which is the case for a 1:2.1 urea/methanol ratio. At smaller ratios the solution remains turbid.

More diluted solutions gave lower recovery of GLA. The optimal final temperature should be between 4 and 0 C. A further decrease of the final temperature was accompanied by a decreased yield. Additional trials showed that the fatty acids could be replaced by their methyl esters as starting materials, an approach which also gave good results.

The process was upscaled to ton scale. In this dimension it was preferable to carry out two fractionation steps to obtain an enrichment of the GLA content

TABLE 5

Solvent Optimization for Urea Fractionation of Fatty Acids[a]

U:S ratio S = 85% Methanol 15% Water	1:1	1:1.5	1:1.6	1:1.7	1:1.8	1:1.9	1:2
% GLA	65.8	73.7	75.3	72.1	68.3	63.4	59.5
Yield (%)	5.1	8.8	11.0	12.6	13.6	14.2	15.5

[a]Partial substitution of methanol by water and variation of the urea (U):solvent (S) ratio.

TABLE 6

Urea Fractionation of Mixtures of Fresh and Recovered Fatty Acids

Ratio of recovered FA to fresh FA	0:100	10:90	25:75	50:50	75:25	90:10	100:0
% GLA before fractionation	16.7	15.8	14.4	12.0	9.7	8.2	7.3
% GLA of the end product	78.3	75.4	75.3	71.9	62.1	58.8	44.6
Yield (%)	13.7	13.0	12.0	10.7	11.0	9.5	10.5

TABLE 7

Fractionation Cycles with Recovered Fatty Acids[a]

No. of cycles	% GLA of the recovered FA	Yield (%)	% GLA of the end product	% GLA of the FA occluded in urea
1	7.0	12.5	66.8	5.4
2	5.4	12.0	66.0	3.0
3	3.0	9.0	67.5	3.2
4	3.2	10.0	61.5	4.2
5	4.2	10.0	63.9	4.6
6	4.6	9.0	68.2	4.6
7	4.6	12.0	59.0	2.8
8	2.8	9.0	68.7	4.9
9	4.9	10.5	66.7	3.6
10	3.6	7.5	71.5	3.1

[a]Each cycle 1:1 mixtures of fresh and recovered fatty acids, with fresh fatty acids containing 16.8% GLA.

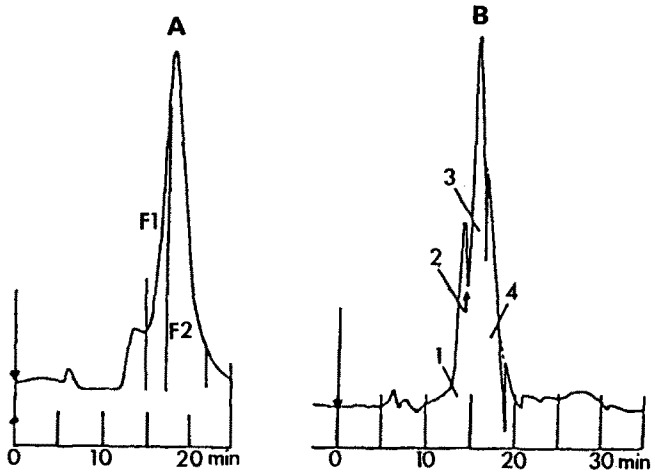

FIG. 2. HPLC chromatograms of GLA concentrates. A, Prep LC/System 500; B, Kiloprep 1010 System.

to about 75%. Increasing quantities rendered the cooling speed during crystallization even more important for the selectivity of the fractionation.

Other experiments confirmed that methanol was the solvent best suited for this process. Partial substitution of methanol by water may be an interesting alternative for reducing solvent quantities, as the solubility of urea is increased in these blends. With a mixture of 85% methanol and 15% water the ratio of urea to solvent could be decreased from 1:2.1 to 1:1.6 with satisfactory results, as shown in Table 5. Further decreasing this ratio also diminshed the GLA content.

Recycling of used fatty acids. About 85% of the fatty acids employed are occluded in the urea, containing still ca. 7% GLA. As BCO is a valuable raw material with limited availability it is important for industrial scale operations that these acids be regenerated for further use. The acids can be released by treatment of the precipitated urea with water. Trials showed that urea, purified from fatty acids and dried, was no longer suitable for use in such fractionations because of diminished selectivity. To test the possibility of reusing fatty acids, a series of fractionations with mixtures of fresh and recovered fatty acids in different ratios was undertaken. The results are shown in Table 6. Here it can be seen that for mixtures of fatty acids, ranging from 100% fresh to equal parts fresh/recovered, enrichment in GLA was in a similar range of 78.3 to 71.9%. Even the fractionation of 100% recovered fatty acids gave an enrichment from 7.3 to 44.6% GLA. Thus it is clear that recovered fatty acids can be refractionated successfully, allowing optimal use of the raw material.

To evaluate how often fatty acids may be reused a series of 10 fractionations was carried out with each time recovered and fresh fatty acids in the ratio 1:1, and each time recycling the fatty acids recovered from the preceding fractionation. These results are shown in Table 7. After 2–3 cycles an equilibrium state is reached, characterized by a GLA content of 60–70% in the final product. This process renders the possibility of decreasing the GLA concentration in waste fatty acids from 7–8% to 3–4%. On the basis of these values it is estimated that any given quantity of final product can be obtained from only 75% of the starting material required by other methods using 100% fresh fatty acids. For this estimation a slightly changed quality of the end product was neglected.

Enrichment in γ-linolenic acid by HPLC. With urea, GLA and stearidonic acid are cofractionated so that no further concentration of GLA can be obtained by a second or third urea fractionation. However, these acids can be separated by preparative liquid chromatography (see Experimental Procedures). Typical HPLC chromatograms of GLA concentrates obtained by urea fractionation are shown in Figure 2, showing chromatograms recorded with a Prep LC/System 500 or a Kiloprep KP 1010 System. Unexpectedly, the shoulder, representing the stearidonic acid, is better separated by the Kiloprep KP 1010 than by the Prep 500 system with a specific loading up to four times higher. The observed differences between these chromatograms should not be due to structural or geometrical effects of the two column types but rather to physicochemical properties of these fatty acids.

The fatty acid compositions of the fractions marked in the chromatograms of Figure 2, determined by GC analysis, are listed in Table 8, demonstrating the good applicability of this method for separating GLA from stearidonic acid and proving the feasibility of a GLA enrichment to > 95% by combining urea fractionation with preparative liquid chromatography. Moreover, this method is also suitable to obtain fractions rich in steari-

TABLE 8

Fatty Acid Composition in %: Starting Material and Samples of HPLC Separations Marked in Corresponding Chromatograms of Fig. 2

		Prep LC/System 500 A		Kiloprep 1010 system B			
Fatty acids	Starting material	F1	F2	1	2	3	4
C18:2	0.2	0.6	0.1	0.3	0.2	0.1	0.6
C18:3γ	80.2	48.2	95.4	19.8	53.4	94.0	95.7
C18:3α	2.5	2.0	3.4	0.7	1.5	2.5	2.5
C18:4	16.6	46.3	0.9	78.3	44.6	3.2	0.9
Others	0.5	2.9	0.2	0.9	0.3	0.2	0.3

donic acid which might be of prime interest as they contain simultaneously biologically active n-6 and n-3 fatty acids in high concentrations. The ratio of the two acids can be mediated by varying the cutting of the HPLC fractions.

In this way GLA fractions or stearidonic acid fractions can be used as pure fatty acids or in the form of triglycerides after reesterification with glycerol, e.g., for dietetic, cosmetic or medical applications. Furthermore, GLA can serve as a raw material for the synthesis of dihomo-γ-linolenic acid.

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