CHARACTERIZATION OF TRIACYLGLYCEROLS IN *ARBUTUS UNEDO* L. SEEDS

CARATTERIZZAZIONE DEI TRIACILGLICEROLI
IN SEMI DI *ARBUTUS UNEDO* L.

M.S. SIMONETTI, F. DAMIANI[1], L. GABRIELLI[1], L. COSSIGNANI, F. BLASI, F. MARINI[2], D. MONTESANO, A. MAURIZI[3], F. VENTURA, A. BOSI and P. DAMIANI[*]

Dipartimento di Scienze Economico-Estimative e degli Alimenti,
Sezione di Chimica Bromatologica, Università degli Studi di Perugia,
Via S. Costanzo, 06126 Perugia, Italy

[1] Dipartimento di Materiali e Risorse Naturali, Università di Trieste,
Via Valerio 6, 34127 Trieste, Italy

[2] Dipartimento di Chimica, Università di Roma "La Sapienza",
P.le Aldo Moro 5, 00185 Roma, Italy

[3] Dipartimento di Medicina Interna, Sezione di Biochimica Applicata
e Scienze della Nutrizione, Università degli Studi di Perugia,
Via S. Costanzo, 06126 Perugia, Italy

*Corresponding author: Tel. +39 075 5857921, Fax +39 075 5857921,
e-mail: chimbrom@unipg.it

ABSTRACT

Arbutus unedo L. (fam: Ericaceae), a typical plant of the Mediterranean woods, produces many reddish berries with small seeds. This study was carried out to characterize the triacylglycerol (TAG) fraction of the oil extracted

RIASSUNTO

Arbutus unedo L. (fam: Ericaceae), tipica pianta della macchia mediterranea, produce numerose bacche rossastre con piccoli semi. La presente ricerca è stata condotta al fine di caratterizzare la frazione triacilglicerolica (TAG)

- Key words: *Arbutus unedo*, Ag^+-HPLC, lipids, stereospecific analysis, triacylglycerols -

from the seeds of *A. unedo* berries by stereospecific analysis and by a procedure based on hyphenated techniques (silver ion high-performance liquid chromatography -Ag^{+}-HPLC- and stereospecific analysis of some sub-fractions). *A. unedo* lipids have a notable amount of essential fatty acids (EFA) and low levels of saturated fatty acids; furthermore there is a marked asymmetry in the FA positional distribution on the TAG glycerol backbone.

dell'olio estratto dai semi delle bacche di *A. unedo* sia mediante analisi stereospecifica sia con hyphenated techniques (tecniche collegate, nel caso specifico costituite da cromatografia di argentazione -Ag^{+}-HPLC- e analisi stereospecifica chimico-enzimatica di alcune sub-frazioni). I lipidi della matrice considerata presentano un significativo contenuto di acidi grassi essenziali (EFA) e bassi livelli di FA saturi; inoltre, è stata rilevata una notevole asimmetria nella distribuzione degli FA nello scheletro glicerolico.

INTRODUCTION

Strawberry tree (*Arbutus unedo* L., fam: Ericaceae) is a typical plant of the Mediterranean woods. Its name is probably attributable to Virgil; in fact it seems that the poet derived the name *Arbutus* from the latin words "*arbustus*" and *unum edo* from "unum edo". Strawberry tree is an evergreen shrub or small tree of average size, with twisting, reddish branches, alternating lanceolate serrate leaves and flowers clustered in racemes at the end of the branches. It produces many spherical reddish berries, with a yellow or orange sweet delicate pulp that tastes like strawberry; the berry contains many small seeds whose composition deserves study (SCORTICHINI, 1986; 1990).

The *Arbutus* genus is distributed throughout southern Europe, but is also present in some areas of Africa, Asia, Canada and northwestern America. *A. unedo* found in the Mediterranean area is a spontaneous species of great landscaping and ecological value (SEIDEMANN, 1995). Regular consumption of strawberry tree berries is desirable, because the composition of the fruit is quite similar to many of the more widely consumed fruits, particularly when the sugar (14% fresh wt) and vitamin C (150-280 mg/100 g fresh wt) contents are considered (AYAZ *et al.*, 2000; ALARCÃO-E-SILVA *et al.*, 2001). Other studies on strawberry tree have reported on the lipid (MELETIOU-CHRISTOU *et al.*, 1994), essential oil (KIVCAK *et al.*, 2001), volatile compound (YAYLI *et al.*, 2001) and flavonoid (MALES *et al.*, 2006) contents of the fruit.

In this study the triacylglycerol (TAG) fraction of the oil extracted from *A. unedo* seeds was studied. The aim was to characterize the TAG fraction, particularly the iso-unsaturated TAG sub-fractions of the strawberry tree seed by stereospecific analysis of the total fraction, as well as by silver ion high-performance liquid chromatography -Ag^{+}-HPLC- and stereospecific analysis of some sub-fractions.

MATERIALS AND METHODS

Materials

Sampling

Strawberry tree (*A. unedo* L.) fruits were obtained from young trees in central Italy (Umbria and Lazio) in November 2005. The berries were firm, spherical, bright scarlet-red, with a diameter of 1-1.5 cm. Five samples of *A. unedo* berries were randomly collected (about 1 kg for each sample). The oil samples were obtained from the milled and homogenized seeds by Soxhlet extraction with *n*-hexane as solvent; the organic extracts were pooled, treated with anhydrous Na_2SO_4 and the organic solvent was evaporated.

Chemicals and reagents

All the solvents and reagents, Analar or HPLC grades, were supplied by Sigma-Aldrich (St. Louis, MO).

Methods

Fatty acid (FA) composition (%) of the TAG fraction by high resolution gas chromatography

The TAG fraction was separated from *A. unedo* seed samples by thin layer chromatography TLC as previously described (NERI *et al.*, 1998), using silica gel plates, 0.25 mm, 20 cm x 20 cm (Macherey-Nagel, Düren, Germany) and a mixture of petroleum ether 40-60°C/diethyl ether/formic acid (70/30/1, v/v/v) as eluent.

The methyl ester derivatives of the constituent fatty acids (FAME) were prepared by sodium methoxide-catalyzed transesterification, in the presence of an internal standard, methyl nonadecanoate, to quantify the sub-fractions, previously separated by Ag^+-HPLC (SANTINELLI *et al.*, 1992). A Chrompack 9001 gas chromatograph (Chrompack International B.V., Middelburg, The Netherlands), equipped with a split/splitless injection system and a flame ionization detector was used for FAME analysis; the separation was obtained using a Supelcowax 10 fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Supelco, Bellefonte, PA). The oven temperature was maintained at 165°C for 3 min, then raised 3°C/min to 240°C and held for 10 min. Helium was the carrier gas at a flow rate of 2 mL/min. A MOSAIC integration software (Chrompack International B.V.) was used for data acquisition and processing.

Ag^+-HPLC analysis

The HPLC analyses were carried out using a solvent delivery system Model LC-10AD VP (Shimadzu, Kyto, Japan) equipped with a light-scattering detector (Model DDL21, Cunow SA, Cergy St. Cristophe, France). The chromatograms were acquired and the data were analyzed using the Shimadzu Class-VP software.

The chromatographic separation of TAG was achieved with a Ag^+ ChromSpher 5 Lipids (5 μm; 250 mm × 4.6 mm) column (Chrompack International B.V.) operating at ambient temperature.

An adjustable stream-splitter was installed between the column and the detector.

The analyses were carried out using a ternary solvent gradient system: (A) 1,2-dichloroethane/dichloromethane (1:1, v/v); (B) acetone; (C) acetone/acetonitrile (9:1, v/v). The analysis conditions were: from 100% A to 60% A - 40% B for 18 min and then to 90% B - 10% C for 25 min; the flow rate was 1.0 mL/min (SANTINELLI *et al.*, 1992).

Stereospecific analysis of TAG

Pancreatic lipase hydrolysis was carried out on TAG to obtain *sn*-2-monoacylglycerols (*sn*-2-MAG), according to the NGD method (1989); the HRGC analysis of FAME of *sn*-2-MAG gave the FA % composition at the *sn*-2- position (A_2). The preparation of *sn*-1,2-phosphatidic acids (*sn*-1,2-PA) was carried out by *sn*-1,2-diacylglycerol kinase procedure (DAMIANI *et al.*, 1994a,b); the HRGC analysis of FAME of *sn*-1,2-PA gave the FA % composition at the *sn*-1,2- positions ($A_{1,2}$).

The FA composition at the *sn*-1- and *sn*-3- positions was obtained using the

% FA compositions of *sn*-1,2-PA, *sn*-2-MAG and total TAG (At), applying the following formulae:

$$A_1 = 2 \cdot A_{1,2} - A_2$$
$$A_3 = 3 \cdot At - A_2 - A_1$$

Statistical analysis

Five replications of each analytical determination were performed. The values are reported as the mean value ± standard deviation.

RESULTS AND DISCUSSION

The TAG fraction, purified from *A. unedo* lipids (11.1% of seeds), was analyzed for its FA composition and the stereospecific distribution. Subsequently, Ag^+-HPLC analysis was used to separate the TAG fraction according to the degree of unsaturation (CHRISTIE, 1982), and the major iso-unsaturated sub-fractions (> 3%) were further characterized.

Table 1 shows the FA composition of the total lipids and the TAG fraction from the five *A. unedo* seed samples. The results show that the total lipids and TAG fraction were made up of low levels of saturated FA (SFA, about 14.0%) and high levels of monounsaturated (MUFA, about 41.1%) and polyunsaturated ones (PUFA, about 45.0%). Strawberry tree seed oil is of particular interest because of its high essential FA (EFA) content, with 18.7% linoleic (L, C18:2n-6) and 26.3% α-linolenic (Ln, C18:2n-3) acids.

Table 1 - Fatty acid composition of total lipids and TAG fraction of *A. unedo* seed oil.

	A. unedo seed oil	
	Total lipids	TAG fraction
C 14:0	0.3±0.07	0.3±0.06
C 16:0	9.7±0.32	9.6±0.15
C 16:1n-7	0.6±0.14	0.7±0.09
C 18:0	3.6±0.19	3.5±0.08
C 18:1n-9	40.1±1.08	40.1±0.12
C 18:2n-6	18.7±0.22	18.6±0.28
C 18:3n-3	26.3±0.29	26.5±0.11
C 20:0	0.4±0.09	0.3±0.04
C 20:1n-9	0.3±0.12	0.3±0.06
SFA	14.0	13.7
MUFA	41.0	41.1
PUFA	45.0	45.1

mean values, mol % ± standard deviations (n=5).

Table 2 - Positional fatty acid composition of TAG fraction from *A. unedo* seed oil.

	sn-1-	*sn*-2-	*sn*-3-
C 14:0	0.4±0.04	0.1±0.00	0.3±0.15
C 16:0	19.3±0.19	0.5±0.11	8.9±0.56
C 16:1n-7	1.1±0.07	0.3±0.13	0.7±0.27
C 18:0	6.8±0.35	0.2±0.05	3.6±0.45
C 18:1n-9	38.4±0.27	49.2±0.15	32.7±0.52
C 18:2n-6	15.2±0.26	22.6±0.07	18.1±0.94
C 18:3n-3	17.7±0.16	26.8±0.13	35.0±0.35
C 20:0	0.3±0.09	0.2±0.04	0.4±0.14
C 20:1n-9	0.7±0.10	0.1±0.02	0.1±0.03
SFA	26.8	1.0	13.2
MUFA	40.2	49.6	33.5
PUFA	32.9	49.4	53.1

mean values, mol % ± standard deviations (n=5).

The FA positional composition of the TAG fraction, obtained from stereospecific analysis, is reported in Table 2. The *sn*-2- position was almost entirely (99.0%) esterified with unsaturated FA, 49.6% MUFA and 49.4% PUFA. Only 1.0% SFA were found at this position, as generally observed in vegetable oils (CHRISTIE, 1987). Oleic acid (O; C18:1n-9) was the most abundant FA (49.2%) in the *sn*-2- position, followed by EFA (L 22.6%; Ln 26.8%).

The results of stereospecific analysis showed an asymmetry in the distribution of the FA between the *sn*-1- and *sn*-3- positions of the TAG fraction. For example, palmitic (P, C16:0) and stearic (S, C18:0) acids had similar

Table 3 - Major iso-unsaturated TAG sub-fractions of *A. unedo* seed oil (S, saturated; M, mono-unsaturated; D, di-unsaturated; T, tri-unsaturated FA).

TAG sub-fraction	mol (%)
MMT	13.5
MDT	12.1
SMT	9.8
MMD	9.2
MTT	8.4
SMM	7.2
SMD	6.7
SDT	4.6
MDD	4.2
DTT	3.7
STT	3.2

mean values, mol %; n=5.

trends, showing a marked preference for the *sn*-1- position. There was a particular positional composition for PUFA; linoleic acid was preferably located in the *sn*-2- position (22.6%) and less in the *sn*-3- and *sn*-1- positions (18.1% and 15.2%, respectively). Linolenic acid was preferably esterified in the *sn*-3- position (35.0%), with 26.8 and 17.7% in the *sn*-2- and *sn*-1- positions, respectively.

Table 3 shows the proportions of the major (> 3%) TAG sub-fractions separated by Ag^{+}-HPLC analysis. The TAG sub-fractions were also quantitatively evaluated by using a home-made software that processes the results of positional composition of FA to provide information about the contents of all the TAG molecular species.

The stereospecific analysis was carried out on the most representative TAG sub-fractions, MMT, MDT, SMT, MMD, MTT and SMM (S, saturated; M, mono-unsaturated; D, di-unsaturated; T, tri-unsaturated FA); their positional compositions are reported in Table 4. A marked asymmetry of the FA distribution in the

Table 4 - Positional fatty acid composition of the iso-unsaturated sub-fractions: MMT, MDT, SMT, MMD, MTT and SMM (S, saturated; M, mono-unsaturated; D, di-unsaturated; T, tri-unsaturated FA).

	MMT			MDT			SMT		
	sn-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C14:0							1.1	0.2	0.5
C16:0							51.7	1.0	17.7
C16:1n-7	2.1	0.4	0.9	1.2	0.2	0.4	0.4	0.3	0.5
C18:0							18.2	0.3	7.1
C18:1n-9	74.9	72.7	47.3	40.7	34.7	21.9	15.1	58.6	24.5
C18:2n-6				33.2	37.5	29.4			
C18:3n-3	21.7	26.7	51.6	24.2	27.5	48.2	12.3	39.0	48.7
C20:0							0.9	0.5	0.8
C20:1n-9	1.3	0.1	0.2	0.7		0.1	0.3	0.1	0.1
	MMD			**MTT**			**SMM**		
	sn-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C14:0							1.0	0.2	0.7
C16:0							44.5	0.9	24.6
C16:1n-7	2.0	0.4	1.2	1.2	0.2	0.4	1.0	0.5	1.2
C18:0							15.7	0.3	9.9
C18:1n-9	79.4	66.3	59.2	42.8	36.1	18.4	36.5	97.5	62.2
C18:2n-6	27.5	33.2	39.4						
C18:3n-3				55.2	63.6	81.2			
C20:0							0.8	0.4	1.1
C20:1n-9	1.2	0.1	0.2	0.7		0.1	0.6	0.2	0.2

three *sn*- positions on the glycerol backbone was observed for all the major subfractions.

The results showed that in the MMT, MDT, MMD and MTT fractions all the MUFA were preferably located at the *sn*-1- position. Oleic acid, the most abundant FA, showed the same trend in all these fractions and was preferentially esterified in the *sn*-1- position, followed by the *sn*-2- and *sn*-3- positions. Oleic acid preferred the *sn*-2- position in the SMT and SMM fractions (58.6% and 97.5%, respectively). PUFA showed a particular distribution in all the cited fractions except for SMM. Linoleic acid was preferably located at the *sn*-2- position (37.5%) in the MDT fraction but it was located at the *sn*-3- (39.4%) in the MMD fraction. Linolenic acid was the most abundant acid in the *sn*-3- position of the MMT, MDT, SMT and MTT subfractions. The SFA in the SMM fraction were located mainly at the *sn*-1- position, followed by the *sn*-3- position.

Some of the molecular isomeric TAG species of the major sub-fractions are shown in Fig. 1. The results showed the presence of different levels of isomeric and enantiomeric TAG species. For example, the ratio of enantiomeric species *sn*-OOLn (6.6%) and *sn*-LnOO (2.8%) was about 2.3 in the MMT sub-fraction. This ratio, together with others of different TAG sub-fractions, could serve as markers to characterize *A. unedo* seed. Furthermore, the TAG species with EFA in the *sn*-2- position could also be of interest from a nutritional point of view. For example, the *sn*-OLnO made up 3.4% of the total TAG in the MMT sub-fraction. In the MDT sub-fraction, about 4.3% of the TAG species (*sn*-OLLn and *sn*-LnLO) had linoleic acid at the *sn*-2- position and about 3.2% of the TAG species (*sn*-OLnL and *sn*-LLnO) had linolenic acid at this position. In contrast in the SMT sub-fraction, about 2.5% of the TAG species (*sn*-PLnO and *sn*-OLnP) had linolenic acid at the *sn*-2-position.

These results support the hypothesis that every natural oil has its own typical FA positional distribution and could be considered as the "fingerprint" of the TAG fraction of the oil. Since dietary lipids are absorbed as *sn*-2-MAG and as free FA, produced by lipase hydrolysis (BRINDLEY, 1985), the positional distribution of FA on the glycerol backbone has an important nutritional impact. Consequently, the stereospecific analysis is also an important tool for determining the nutritional quality of TAG. Furthermore, the enantiomeric ratios between the TAG molecular species could be of considerable importance as markers to distinguish the lipids derived from different species and varieties.

When the composition of strawberry tree seed oil was compared with that of blueberry (*Vaccinium corymbosum*, fam: Ericaceae) (PARRY *et al.*, 2005), *A. unedo* seed oil had a higher oleic acid content, lower linoleic acid content and a similar linolenic acid level, with a n-6/n-3 PUFA ratio of about 0.7. This ratio is the lowest one, if compared with those of other berries. Since the recommended n-6/n-3 FA ratio is estimated to be 4/1, while the current dietary n-6/n-3 FA ratio is about 10/1 (KRIS-ETHERTON *et al.*, 2000), new dietary sources with low n-6/n-3 PUFA ratios are in high demand for improving human nutrition.

CONCLUSIONS

The present study shows that strawberry tree seed oil is characterized by a low SFA content, a high oleic acid content, a significant presence of n-6 and n-3 EFA and a low n-6/n-3 FA ratio. The results of stereospecific analysis of the TAG fraction show marked asymmetry in the FA distribution among the three *sn*-positions. The high incorporation of EFA in the *sn*-2- position is very important from a nutritional point of view. The Ag^+-HPLC analy-

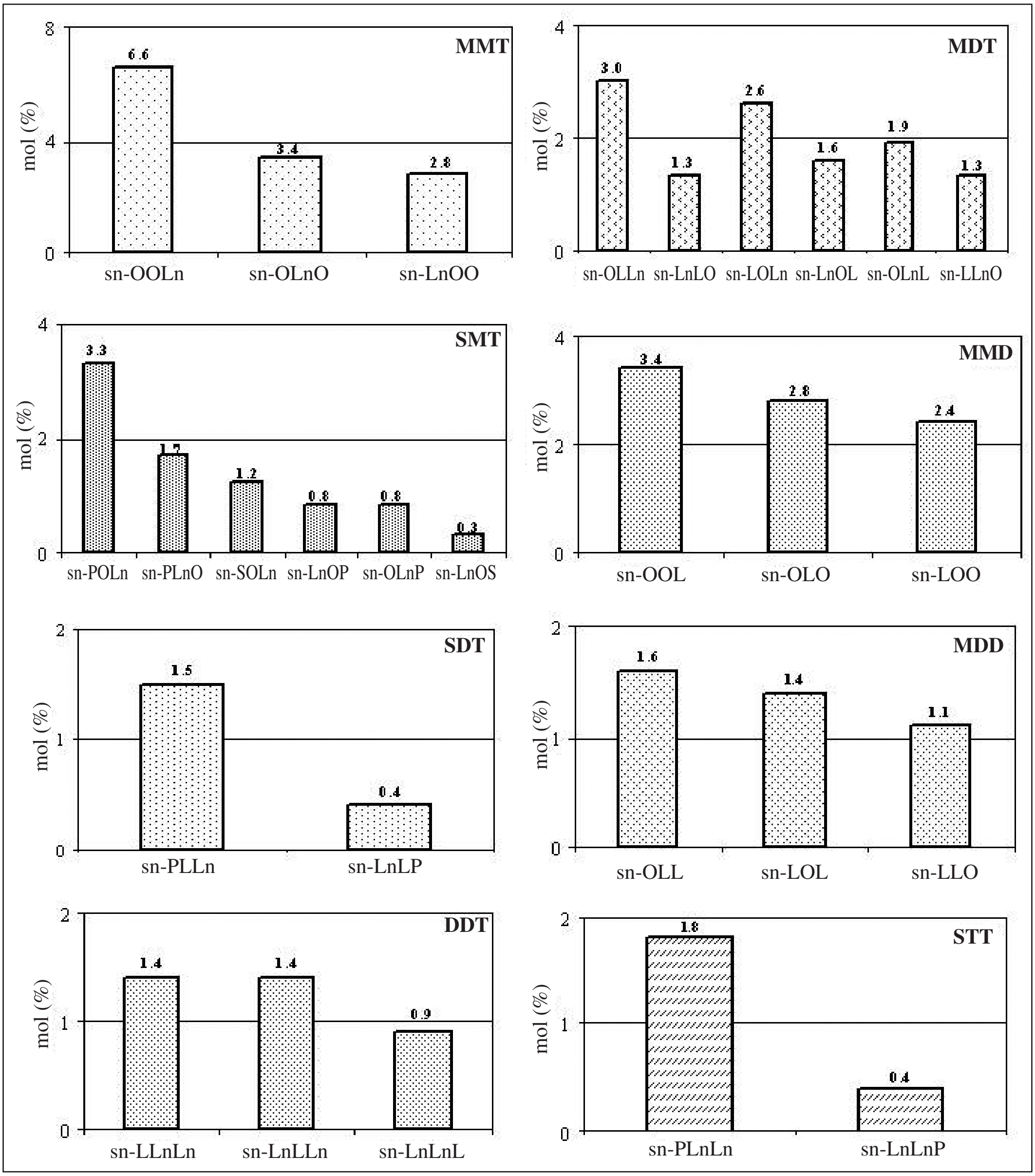

Fig. 1 - Major TAG molecular species of iso-unsaturated TAG sub-fractions of *A. unedo* seed oil: MMT, SMT, SDT, DTT, MDT, MMD, MDD and STT (S, saturated; M, mono-unsaturated; D, di-unsaturated; T, tri-unsaturated FA).

sis of TAG, coupled with stereospecific analysis give other structural information about the lipid fraction. These data could be used to select specific iso-unsaturated sub-fractions, important for their nutritional, physiological and analytical aspects and for industrial applications.

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