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Rapid, micro-scale preparation and very fast gas chromatographic separation of cod liver oil fatty acid methyl esters

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Abstract

The present research is focussed on the optimization of a fast and economically convenient method for the sample preparation and gas chromatographic separation of fatty acids contained in lipidic samples. This, as part of a wider project that has, as ultimate goal, the automation of this specific analytical procedure. The developed approach was applied to the analysis of cod liver oil, a highly complex lipid characterized by a wide nutritional interest. Derivatization was carried out by using low quantities (μL amounts) of a reagent and solvent, while the derived fatty acid methyl esters (FAMEs) were separated in 120 s on a 10 m × 0.1 mm i.d. polar micro-bore column. The total analysis time required for six samples was approximately 45 min (7.5 min/sample), considering a simultaneous process of methylation and GC separation of previously prepared samples. The results obtained were compared to those derived from conventional applications on the same sample. With regard to the validation of the rapid method, peak area/retention time repeatability, linear range, limit of detection (LOD) and quantitation (LOQ) were determined. Peak assignment was carried out by exploiting bidimensional group-type mapping information obtained in a comprehensive gas chromatographic application.

Keywords: Cod liver oil; Polyunsaturated fatty acids (PUFA); Fatty acid methyl esters; Very fast gas chromatography; Comprehensive GC

1. Introduction

In the last decades, increasing scientific emphasis has been laid upon the importance of a correct consumption of dietary fatty acids (FAs). In particular, it is widely accepted that an excessive intake of saturated fatty acids (contained in high amounts in animal-derived fats) and/or a low intake of ω3 polyunsaturated fatty acids (PUFA) may lead to a series of severe metabolic disorders [1–3].

In general, fish is considered an important source of essential fatty acids, which are fundamental from a physiological viewpoint [4]. Cod liver oil, in particular, is a well-known "nutraceutical" and is widely consumed especially in Northern European countries. This food contains an abundant amount of specific vitamins (A, D and E) and ω3 PUFAs [especially eicosapentanoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3)] and, due to this, is used as a dietary supplement [5].

The study of lipidic fatty acids is considered essential for research, clinical and quality control applications [6]. Consequently, the availability of rapid and effective analytical methods in this specific field is of upmost importance for both scientific and industrial communities. The determination of FA profiles in lipids is carried out in two analytical steps; in the first, the matrix is subjected to either basic or acidic derivatization in order to attain the more volatile fatty acid methyl esters (FAMEs). Several procedures and their relative advantages/disadvantages have been described [7,8]. The second step, which consists in the gas chromatographic separation of the derived FAMEs, is generally carried out by using polar conventional capillary columns (25–30 m length; 0.25–0.32 mm i.d.), with separation times generally ranging between 20 and 60 min, depending on the complexity of the lipid [7,8]. The length of such GC runtimes may be considered as a drawback if a high number of samples per day need to be analyzed.

The employment of micro-bore columns in gas chromatography is probably the most efficient way of increasing analysis speed, as they enable the preservation, if compared to conventional approaches, of sufficient separating power for the resolution between the components of interest [9,10].

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The main drawbacks linked to the use of these capillaries are a reduced sample capacity and more drastic instrumental requirements. The former aspect is essentially counterbalanced by a substantial decrease of column band broadening, while with regards to the second issue, modern GC systems guarantee fast GC experimental conditions.

The aim of the present investigation was the optimization of both a low-costing (and rapid) sample preparation process and a very fast micro-bore GC separation step. This, as the preliminary part of a project that has, as final objective, the development of an entirely automated procedure both for very high lipidic sample throughput analysis and for the reduction of manual-intervention related analytical errors. It must be added that, prior to the present FAME automation project, the authors have carried out research in the field of faster lipidic assays [11]; the results attained in the present investigation, though, represent a substantial improvement in all analytical aspects.

Finally, it must be noted that peak assignment was carried out by means of comprehensive two-dimensional gas chromatography (GC × GC) without the support of mass spectrometric data or of pure standard components. The formation of specific chemical class-type patterns on bidimensional (2D) chromatograms is a valuable source of information, that may be exploited for reliable compound identification [12].

2. Experimental

2.1. Samples and sample preparation

The cod liver oil was purchased from a chemist located in Messina (Italy). The pure standard components (methyl hexadecanoate and methyl oleate) were purchased from Larodan Fine Chemicals (Malmö, Sweden).

The cod liver oil FAMEs were prepared as follows: 20 μL of cod liver oil were transesterified in a Pyrex tube by using 200 μL of boron trifluoride-methanol (20% BF_3) reagent (Merck, Milan, Italy) and heating at 100 °C for 30 min. After cooling, 200 μL of *n*-hexane and 800 μL of distilled water was added to the mixture, which was then agitated manually for 1 min and centrifuged for 2 min. Approximately 100 μL of the upper *n*-hexane layer were transferred to a 150 μL glass insert for 2 mL vials (Supelco, Milan, Italy).

Two 10,000 ppm (100 mg/10 mL) stock solutions of standard methyl hexadecanoate and methyl oleate were prepared using *n*-hexane and stored at 4 °C. The rapid method linearity, limit of detection (LOD) and quantitation (LOQ) were determined by using serial dilutions of the stock standard solutions.

2.2. Conventional and very fast gas chromatography

In all applications, the GC system consisted of a Shimadzu GC 2010 equipped with a split–splitless injector (280 °C), AOC-20i autoinjector, AOC-20s autosampler and flame ionization detector (FID) (280 °C) (Milan, Italy). All data were collected by the GC Solution software (Shimadzu, Milan, Italy).

Conventional GC conditions: an Omegawax 250 [100% poly(ethylene glycole)], 30 m × 0.25 mm i.d., 0.25 μm film thickness (Supelco) column was temperature programmed as follows: 180–270 °C (5 min) at 3 °C/min. Injection volume and mode: 0.2 μL; split (50:1). Hydrogen was used as carrier gas at constant linear velocity (35 cm/s) with an initial head pressure of 64.9 kPa. The following gases were used for the FID system: make-up gas was N_2 at a flow rate of 50 mL/min; the H_2 flow rate was 50 mL/min; the air flow rate was 400 mL/min. FID filter time constant and sampling rate: 200 and 80 ms.

Very fast GC conditions: an Omegawax [100% poly(ethylene glycole)], 15 m × 0.1 mm i.d., 0.1 μm film thickness (Supelco) column, was shortened by 5 m and was temperature programmed as follows: 180–270 °C (0.5 min) at 40 °C/min. Injection volume and mode: 0.2 μL; split (200:1). Hydrogen was used as carrier gas at constant linear velocity (100 cm/s) with an initial head pressure of 437.6 kPa. The same gas flows as in the conventional application were used for the FID system. FID filter time constant and sampling rate: 20 and 12 ms.

2.3. Comprehensive two-dimensional gas chromatography

The GC system, which was the same as reported in Section 2.2, was equipped with a LMCS Everest longitudinally modulated cryogenic system (LMCS; Chromatography Concepts, Doncaster, Australia), with a mechanical stepper motor drive for movement of the cryotrap. A 6 s modulation period was applied and initiated by the GC Solution programmed external events that via the electronic controller also started the motor operation. CO_2 was supplied to the trap, and its expansion cooled the trap that was thermostatically regulated at about −10 °C. A small internal flow of nitrogen gas (about 10 mL/min) prevented ice formation inside the trap. Data were collected by the GC Solution software and by using its export function; the ASCII data were converted into a matrix with rows corresponding to a 6 s duration, and data columns covering all successive second dimension 6 s chromatograms using the Comprehensive Chromatography Converter 1.0 software (Università di Messina, Italy). Contour representation of the 2D chromatograms was achieved through the same software. The column set consisted of two capillaries, which were serially connected by a zero-dead-volume press-fit (Mega, Legnano, Italy). The conventional first dimension was an Equity-5 (5% diphenyl + 95% dimethyl polysiloxane) 30 m × 0.25 mm i.d., 0.25 μm film thickness column (Supelco) and the secondary fast column was a Supelcowax-10 [100% poly(ethylene glycole)], 1 m × 0.25 mm i.d., 0.25 μm film thickness (Supelco). The column set was temperature programmed from 180 to 280 °C (10 min) at 3 °C/min. Injection volume and mode: 0.2 μL; split (200:1). Hydrogen was used as carrier gas at constant linear velocity with an initial head pressure of 75.4 kPa. The same gas flows as in the previous applications were used for the FID system. FID filter time constant and sampling rate: 20 and 12 ms.

3. Results and discussion

Initially, a micro-scaled rapid derivatization procedure was optimized and applied to a 20 μL cod liver oil sample (see Sec-

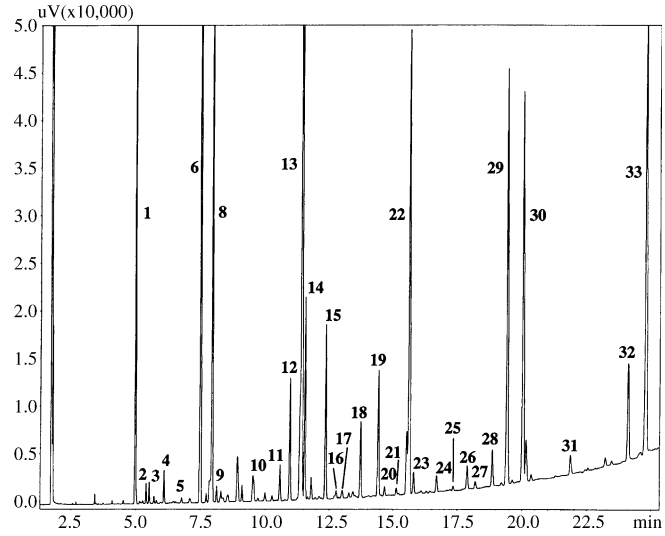

Fig. 1. Conventional GC analysis of cod liver oil FAMEs. Peak identification: (1) C14:0; (2) C15:0 anteiso; (3) C15:0 iso; (4) C15:0; (5) C16:0 iso; (6) C16:0; (7) C16:1ω9; (8) C16:1ω7; (9) C16:1ω5; (10) C16:3ω4; (11) C16:4ω4; (12) C18:0; (13) C18:1ω9; (14) C18:1ω7; (15) C18:2ω6; (16) C18:2ω4; (17) C18:3ω6; (18) C18:3ω3; (19) C18:4ω3; (20) C18:4ω1; (21) C20:0; (22) C20:1ω9; (23) C20:1ω7; (24) C20:2ω6; (25) C20:3ω6; (26) C20:4ω6; (27) C20:3ω3; (28) C20:4ω3; (29) C20:5ω3; (30) C22:1ω9; (31) C21:5ω3; (32) C22:5ω3; (33) C22:6ω3.

tion 2.1). The total consumption of the reagent and solvent was low, corresponding to 200 μL each of boron trifluoride-methanol and *n*-hexane. About 100 μL of derivatized matrix were attained from the final mixture, and thus, the employment of a 150 μL glass vial insert was necessary for automated GC injection. The time required for the preparation of a single sample was about 35 min. Five sequential samples were methylated and analyzed by means of conventional GC (unless stated, all the following data reported in this paper derive from five consecutive analysis), under optimum experimental conditions (see Section 2.2). The GC separation, relative to the last application of the series and carried out in about 25 min, is shown in Fig. 1. The 33 peaks numbered in the figure (identification is reported in the caption)

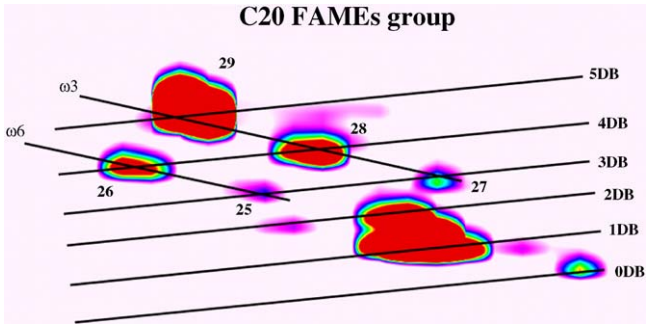

Fig. 2. Expansion of the GC × GC cod liver oil FAMEs (C20 group) 2D chromatogram. Peak identification as in Fig. 1.

were assigned through comprehensive GC data. This choice was related to the fact that FAME-differentiation, by means of GC–MS, is rather complicated because many esters are characterized by similar fragmentation patterns. Furthermore, not all of these compounds are available as pure standard components. It must be emphasized that the 2D chromatographic profile of the sample is much more complex than what can be expected by observing Fig. 1. A vast number of ultra trace-amount compounds (especially in the C15, C17, C19, C21 FAMEs groups), which are either below the conventional GC limit of detection or overlap with one or more components, were clearly visible in the two-dimensional chromatogram. As demonstrated in previous GC × GC research on a complex fish oil [13], esters within each FAME class and characterized by the same number of double bonds tend to align themselves along slanting horizontal bands; the same occurs, along slanting vertical bands, for FAMEs with the same ω number. These ordered elution patterns can be exploited for reliable peak assignment: diagonals can be drawn through components characterized by the same number of double bonds and through those with the same ω number; the points of intersection correspond to a specific ester, the identity of which may be easily deduced. An example of this procedure, exploited for the assignment of C20 FAMEs, is reported in a 2D space plane expansion, illustrated in Fig. 2.

Table 1
Relative standard deviation values, derived from five consecutive applications, for conventional GC (abbreviated as Conv.) and very fast GC (abbreviated as Very fast) retention times (t_R) and peak areas, as well as conventional and very fast GC mean relative percentage peak areas (% areas)

Peak	RSD (%) Conv. t_R	RSD (%) Conv. areas	RSD (%) Very fast t_R	RSD (%) Very fast areas	Conv. % areas	Very fast % areas
1	0.35	1.15	0.11	2.46	5.29	5.09
3	0.34	2.57	0.08	2.23	0.22	0.20
5	0.33	1.34	0.07	5.83	0.08	0.08
6	0.32	1.21	0.06	2.78	12.47	13.99
11	0.26	1.77	0.06	3.65	0.55	0.50
12	0.25	1.57	0.07	3.16	1.79	1.79
15	0.23	0.99	0.08	4.01	2.71	2.64
18	0.20	0.33	0.07	3.99	1.26	1.21
19	0.20	0.37	0.07	4.19	2.09	1.92
20	0.19	2.22	0.06	3.36	0.16	0.16
21	0.18	2.32	0.12	4.96	0.12	0.12
25	0.15	5.84	0.05	5.55	0.10	0.08
28	0.14	0.90	0.05	3.10	0.67	0.61
31	0.11	6.16	0.05	6.58	0.39	0.35
33	0.08	1.64	0.04	4.20	9.23	10.18

Conventional GC intra-assay retention time and peak area repeatability were determined; RSD values, relative to 15 of the 33 identified components located across the entire chromatogram, are reported in Table 1. As it can be seen, RSDs for retention times are quite satisfactory (0.35% was the maximum value), while those for peak areas are very good, with 13 compounds under 3%; slightly higher but still acceptable values were attained for peak 25 (5.84%), a trace-amount compound, and peak 31 (6.16%). LODs, which were determined by analyzing standard C16:0 and C18:1ω9 and by measuring the mean noise value (sample blanks were injected) plus 3 standard deviations of the blank mean, were 32 and 36 pg, respectively. LOQs, which were determined for the same pure standard compounds by measuring the mean noise value plus 10 standard deviations of the blank mean, equalled 72 and 83 pg, respectively.

At this point, a micro-bore column very fast GC method, based on an accelerated temperature program rate (40 °C/min) and rather high gas linear velocity (100 cm/s), was applied to the cod liver oil FAMEs (see Section 2.2). The GC separation, which was achieved in about 120 s, is illustrated in Fig. 3. As it can be observed, the quality of the chromatographic separation may be considered as very good, especially considering that the micro-bore column was operated at far from both the optimum temperature rate (in the 10–12 °C/min range) and gas linear velocity (in the 50–55 cm/s range); this has been defined in previous (unpublished) work. It is obvious that such a drastic reduction of the GC run-time cannot be attained without a cost, in terms of peak resolution (R_S). Of the 33 identified peaks, only the critical pairs 13–14 (C18:1ω9–C18:1ω7) and 22–23 (C20:1ω9–C20:1ω7), which were separated in the conventional application, underwent different degrees of partial coelution, as can be observed in the conventional and very fast chromatogram expansions, reported in Fig. 4a and b, respectively. In order to compare resolution between the two applications, R_S values were calculated for peak pairs 2–3, 18–19 and 26–27, equalling 2.3, 7.7 and 3.2 in the conventional analysis and 1.9, 4.4 and 1.7 in the fast application. The total GC run to run-time was about

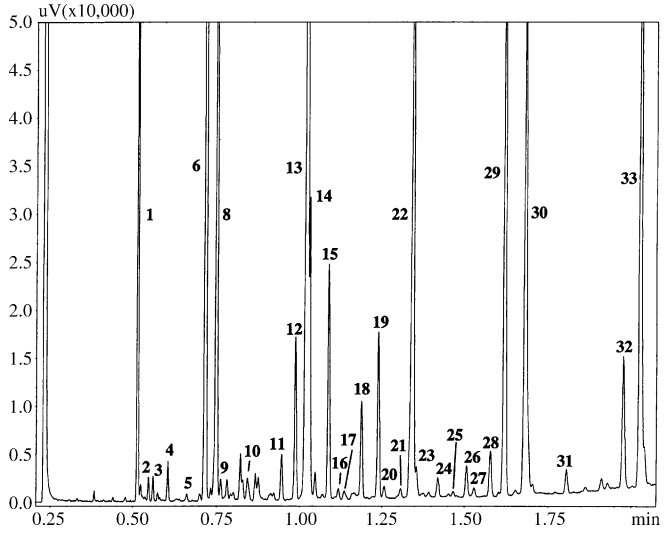

Fig. 3. Very fast GC analysis of cod liver oil FAMEs. Peak identification as in Fig. 1.

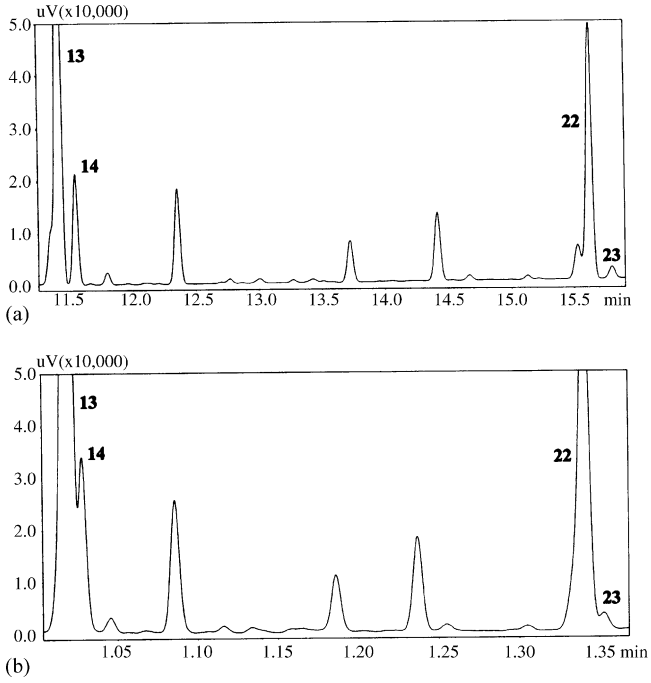

Fig. 4. Expansions of the conventional (a) and very fast (b) chromatograms illustrating the critical pairs 13–14 and 22–23.

6 min and was measured considering 73 s from the start signal to GC injection (transport time of the vial from the autosampler tray to the autoinjector, plus two syringe rinses with pure solvent and one with the sample), the total GC run-time: 2.75 min, the GC cooling time: 51 s and again 73 s from the start signal to GC injection. It must be emphasized that, in order to maintain the column in good operational conditions, a blank sample must be run every six applications.

It is obvious that in a high sample throughput automated system, sample preparation will be carried out simultaneously on a series of matrices; this, while previously prepared samples are sequentially gas chromatographed. It is therefore important that both steps are achieved in about the same time. In order to emulate such an approach, six cod liver FAMEs samples (plus a blank) were separated by means of batch GC analysis; the seven applications were carried out in about 42 min. During the GC applications, a further six samples were prepared manually at the same time, requiring approximately 45 min. Considering this, the total analysis time for a single cod liver oil sample was only 7.5 min. Although the same approach was not applied using conventional GC, it was easily derived that the total analysis time for each sample would have been approximately 38 min (the seventh blank sample was not considered).

Very fast GC intra-assay retention time and peak area precision were determined. RSDs relative to the same 15 analytes are reported in Table 1. RSDs for retention times were excellent (0.12% was the maximum value) and unexpectably better than in the conventional GC analysis; the precision for peak areas, although a little higher than in the conventional application, was generally satisfactory (RSDs for all compounds were under 7%). It may be affirmed that the application of more severe experimental parameters did not have a negative effect on the analytical

performance. Mean relative percentage peak areas (% areas), measured in both applications, are also reported in Table 1. As it can be observed, the two methods are in good agreement as there are only negligible differences between the mean % areas. LODs, which were determined through analyses carried out on C16:0 and C18:1ω9 as previously described, were 21 and 22 pg, respectively, while LOQs equalled 40 and 45 pg, respectively. The higher method sensitivity is due to the fact that micro-bore columns are characterized by reduced resistance to mass transfer in the mobile phase, hence band broadening is greatly limited and narrower peaks are generated. It must be added though, that the detector parameters (see Section 2.2) applied, which were necessary for both adequate peak re-construction and resolution, generated a substantial degree of baseline noise, thus causing a slight decrease in sensitivity. The mean blank noise values were, in fact, 58 and 17 μV in the very fast and conventional applications, respectively. Although various opinions exist, it is generally accepted that 15–20 data points per peak are required for correct compound quantification [9]. The more rapidly eluting peaks in Fig. 3 were characterized by a base peak width in the 300–350 ms range; hence, a sampling rate of 12 ms (83 Hz) was employed. Finally, the rapid method linear detection range was evaluated over 3 orders of magnitude (0.1–100 ng) for C16:0 and C18:1ω9, with regression coefficients (R^2) equal to 0.9993 and 0.9967, respectively.

4. Conclusions

As aforementioned, this work may be considered the primary "easier" step towards the development of a fully automated approach for the analysis of naturally occurring and industrially produced lipids. The fast method here described, provided a satisfactory result in all of the reported analytical aspects and proved to be more convenient than a widely applied conventional method. Future research will be devoted to automation, which will hopefully be achieved by using an autosampler combined with the GC instrumentation used in this investigation. The autosampler will be partly constructed according to specific specifications related to the derivatization procedure.

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References

[1] F.M. Sacks, M. Katan, Am. J. Med. 113 (2002) 13–24.
[2] R.N. Lemaitre, I.B. King, D. Mozaffarian, L.H. Kuller, R.P. Tracy, D.S. Siscovick, Am. J. Clin. Nutr. 77 (2003) 319–325.
[3] D.S. Siscovick, T.E. Raghunathan, I. King, S. Weinmann, V.E. Bovbjerg, L. Kushi, L.A. Cobb, M.K. Copass, B.M. Psaty, R. Lemaitre, B. Retzlaff, R.H. Knopp, Am. J. Clin. Nutr. 71 (2000) 208S–212S.
[4] H.-D. Belitz, W. Grosch, Food Chemistry, Springer-Verlag, Berlin, 1999.
[5] M. Brustad, T. Braaten, E. Lund, Eur. J. Clin. Nutr. 58 (2004) 128–136.
[6] T. Seppänen-Laakso, I. Laakso, R. Hiltunen, Anal. Chim. Acta 465 (2002) 39–62.
[7] K. Eder, J. Chromatogr. B 671 (1995) 113–131.
[8] W.W. Christie, Lipid Analysis, The Oily Press, Bridgewater, England, 2003.
[9] P. Korytár, H.-G. Janssen, E. Matisová, U.A.T. Brinkman, Trends Anal. Chem. 21 (2002) 558–572.
[10] L. Mondello, P.Q. Tranchida, A. Casilli, O. Favoino, P. Dugo, G. Dugo, J. Sep. Sci. 27 (2004) 1149–1156.
[11] L. Mondello, A. Casilli, P.Q. Tranchida, R. Costa, B. Chiofalo, P. Dugo, G. Dugo, J. Chromatogr. A 1035 (2004) 237–247.
[12] J. Dallüge, J. Beens, U.A.T. Brinkman, J. Chromatogr. A 1000 (2003) 69–108.
[13] L. Mondello, A. Casilli, P.Q. Tranchida, P. Dugo, G. Dugo, J. Chromatogr. A 1019 (2003) 187–196.