A novel HPLC-ESI-Q-ToF approach for the determination of fatty acids and acylglycerols in food samples

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ABSTRACT: We propose a new analytical method using reverse phase High performance liquid chromatography (HPLC) coupled through an electrospray source with a tandem quadrupole-time-of-flight (ESI-Q-ToF) mass spectrometric detector for the full characterization and quantitation of the different classes of fatty acids and acylglycerols in lipid samples in a single chromatographic run. In this work, we optimized the derivatization reaction for free fatty acids with 2-hydrazinoquinoline, which is a low-cost approach, using a full factorial design. This reaction does not involve transesterification, thus enabling the free fatty acids to be separated and successfully quantified in the presence of mono-, di- and triacylglycerols without altering the whole glyceride profile. This new analytical method provides a full profile of fatty acids, mono-, di- and triglycerides within a relatively short chromatographic run (less than 40 min), with low operating back-pressure (less than 110 bar). The derivatization of the free fatty acids allows their detection in positive mode, with limits of detection in the range of 0.2-1.9 ng/g, and a dynamic range of two orders of magnitude. The figures of merit of the procedure are competitive with respect to the literature.

The method was validated by characterizing two different types of olive oils. Free fatty acid content was quantified, and the results are consistent with literature data. The method was applied to the characterization of cow milk and an infant formula, after the precipitation of proteins and phospholipids, and proved suitable for the detection of short chain fatty acids, free fatty acids and glycerides highlighting differences in the composition of the two milks.

The proposed procedure improves the current methods for the analysis of acylglyceride based materials, such as olive oil, and proved promising for the characterization of lipids in complex matrices, such as milk.

1. Introduction

Oils and fats are complex mixtures of several organic species, mainly belonging to the class of triacylglycerols (TAGs): a glycerol backbone esterified with saturated, unsaturated fatty acids (FAs), or a mixture of both. A relatively small faction (0.5-2%) of free fatty acids (FFAs) is generally also present, together with partially hydrolyzed glycerides: diacylglycerols (DAGs) and monoglycerides (MAGs), with an average composition related to the constituents of the TAGs. Fats and oils have a variety of uses, ranging from food applications to industrial uses, as ingredients in pharmaceutical and cosmetic applications, as paint binders, lubricants, biodiesel, etc, and are being produced in ever-increasing quantities [1]. Growing attention is being paid to the analysis of lipids, for a variety of reasons, including quality, trade and authenticity control [2, 3]. Thus, complete profiling of lipids by chromatographic techniques represents a fundamental tool in the analysis of oils and fats.

Lipidomics research has recently started to develop new and robust procedures for the detection of several classes of lipids in a single sample by liquid chromatography coupled to tandem mass spectrometry. Although several chromatographic methods for the simultaneous analysis of all the lipid classes (FFAs, DAGs and TAGs) have been described, these do not generally entail the quantification of free fatty acids[4, 5]. When the quantification of FFAs or total fatty acids is required, specific methods have been optimized, involving the application of gas or liquid chromatography, often after suitable derivatization reactions [6].

The free or total fatty acid content after saponification of the glyceridic bonds is commonly profiled and quantified by gas chromatography interfaced with mass spectrometric detection. The most common procedures involve the derivatization of acidic moieties by methylation or silylation reactions [7-12]. Gas chromatography is also suitable for the analysis of mono- and diglycerides; however it cannot be used to determine the total lipid profile due to the non-volatility of TAGs [13]. The first chromatographic attempts to characterize the total lipid profile of a material were based on the use of high temperature gas chromatography with a flame ionization detector (HTGC-FID) [14]. HTGC can also be coupled with mass spectrometry (HTGC-MS), thereby identifying the triglycerides by mass spectra interpretation; however it is limited by the decomposition of TAGs at high temperatures [15-17].

The profiling and semi-quantitative analysis of acylglycerols is more commonly performed by HPLC analysis, involving the separation of the analytes on a reverse phase column, interfaced with UV spectrophotometric, evaporative light scattering detection, charged aerosol detector (CAD), or MS detection [8, 14, 18-27].

Although fatty acids are detectable by mass spectrometers operating in negative mode, in the same chromatographic run as other lipid materials, their quantification by HPLC is often performed after suitable derivatization reactions, in order to increase retention times, and to enable to use spectrophotometric detectors (e.g. derivatization with UV-Vis absorbing or fluorescent moieties). These methods are particularly useful when short-chain fatty acids are analyzed. The derivatization reactions are applied to the free fatty acid fractions of the samples, or to a saponified extract [28-30].

This literature overview shows that free fatty acids and acylglycerides of a lipid material are most commonly characterized using two different analytical procedures: usually a gas chromatographic method for the fatty acids analysis, and liquid chromatography for the acylglycerides [22, 31].

The current analytical approaches have several limitations: the complex sample pretreatments can lead to contamination of the sample and to long analysis times, and the use of different analytical instrumentations involves high costs for a single analysis. The analyst also has to be an expert in the use of several analytical instrumental set-ups, thus often making these approaches disadvantageous.

Whenever the simultaneous detection of fatty acids and glycerides is described, along with the evaluation of the total lipidome, the chromatographic conditions often involve very long analysis times, complex elution mixtures, and more than one chromatographic column in series, thus leading to high back-pressures, requiring the use of ultra-pressure LC systems (UPLC)[28-30, 32].

In this paper, we propose a new analytical method for the full characterization and quantitation of glycerides and free fatty acids in lipid-containing samples by reverse phase HPLC coupled with ESI-Q-ToF mass spectrometric detection. Their simultaneous determination allows for shorter analysis times and lower risk of sample contamination, eventually leading to a higher analytical throughput. The use of high resolution mass spectrometry allows us to unambiguously characterize the MAGs, DAGs, and TAGs species, and to overcome possible co-elution of target analytes. We exploited a low-cost derivatization reaction with 2-hydrazinoquinoline, which was recently intro-

duced for the derivatization of short chain carboxylic acids, aldehydes and ketones in biological samples [33]. We tested the reaction in the presence of acylglycerides to evaluate the absence of transesterification or other side reactions, and we optimized the derivatization conditions using a full factorial experimental design. The method was fully validated, showing detection limits and dynamic ranges superior to those achieved by analysis of the underivatized sample solution and working in the negative mode.

The method was tested on a selection of olive oil and milk samples. This new analytical method enabled us to obtain the full lipid profile of the fats in two types of olive oil; our results are qualitatively and quantitatively consistent with literature data. Moreover, we tested the suitability of the procedure for the characterization of cow milk and infant formula, after the precipitation of proteins and phospholipids. The method proved suitable for the detection of short chain fatty acids, free fatty acids and glycerides, as described in the literature. Our results were obtained within a relatively short chromatographic run (less than 40 min) and with low back-pressure (less than 110 bar), thus enabling us to separate and quantify the free fatty acids in the presence of mono-, di- and triacylglycerols, with a standard HPLC setup and with extremely low detection limits.

2. Materials and methods

2.1. Chemicals

The solvents used as eluents were iso-propanol, water and methanol (HPLC-MS grade; Fluka). A stock fatty acid solution was prepared in acetone (HPLC grade; Sigma-Aldrich), and stored at 4 °C in the dark. It contained lauric acid (0.21 μg/g), suberic acid (0.22 μg/g), azelaic acid (0.16 μg/g), myristic acid (0.16 μg/g), sebacic acid (0.17 μg/g), palmitic acid (0.19 μg/g), oleic acid (0.26 μg/g), linoleic acid (0.20 μg/g), linolenic acid (0.18 μg/g) and stearic acid (0.26 μg/g). This solution was used to derive the calibration curves in the concentration range 3-400 ng/g. The acids were purchased from Sigma-Aldrich (purity > 99%). A second standard mixture solution was prepared in iso-propanol, containing the following commercial acylglycerols (all purity > 99%, Sigma-Aldrich, U.S.A.): monopalmitin (7.9 μg/g of mP), dipalmitin (7.0 μg/g of PP), trimyristin (8.0 μg/g of MMM), tripalmitin (10.0 μg/g of PPP), and tristearin (7.9 μg/g of SSS). For derivatization, 2-hydrazinoquinoline (HQ), triphenylphosphine (TPP, purity 99%), and 2,2'-dipyridyl disulfide (DPDS, purity 98%) were purchased from Sigma-Aldrich (USA); solutions of 70.20 μg/g HQ, 69.00 μg/g DPDS and 79.50 μg/g TPP were prepared in acetonitrile (LC-MS grade, Sigma Aldrich) and stored at 4 °C in the dark.

2.2. Oil and milk samples

Commercial extra virgin oil, sansa olive oil (low quality oil), whole cow milk, and infant milk formula were purchased at the local super-market. The infant formula label reported that the product was obtained by mixing whole cow milk, coconut oil, rapeseed oil and sun-flower oil.

Oil samples were diluted in acetone 1 to 100 (v/v) prior analysis. The milk samples where diluted in acetone (1:40, v/v), centrifuged and filtered to remove the precipitated proteins and phospholipids [34, 35].

2.3. Derivatization of free fatty acids

The derivatization reaction was optimized on standard solutions of fatty acids starting from the conditions described in [33] for short-chain carboxylic acids, aldehydes and ketones. Carboxylic acids are activated by 2,2'-dipyridyl disulphide (DPDS) and triphenylphosphine (TPP), forming acetylphosphonium ions, which in turn react with 2-hydrazinoquinoline (HQ) to form hydrazides. For the derivatization reaction, 20 μL of standard mixtures or acetone solution of the samples were dried under nitrogen flow in the insert of a 2 mL auto-sampler vial, dissolved with 60 μL of acetonitrile and added with 20 μL of each of the HQ, DPDS, and TPP containing solutions.

The derivatization conditions were optimized using a full factorial experimental design (FFED) with two factors and three levels, selecting as factors the reaction time (t) and the temperature (T) according to the model function $R = b_0 + b_1t + B_2T + b_3\,tT + b_4t^2 + b_5T^2$ (see following subsections for details). FFED was carried out using five monocarboxylic acids as analytes. The conditions tested are reported in Table S1. Water bath and microwave heating were both tested, and the stability of the derivatives was evaluated. Since a low stability and unacceptable reproducibility of the reaction yield was observed when derivatization was carried out in microwave, only the results obtained with the water bath are herein reported.

Data were acquired in triplicates, and chromatographic response (peak areas of the $[M+H]^+$ extracted ions divided by the concentration of the corresponding species) were the response factors used for data analysis.

2.4. *HPLC-ESI-Q-ToF instrument and chromatographic operating conditions*

All the analyses were carried out on a 1200 Infinity HPLC coupled by a Jet Stream ESI interface with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity Q-ToF detector (Agilent Technologies, USA). The chromatographic separation was performed on an Agilent Poroshell 120 EC-C18 column (3.0 mm × 50 mm, 2.7 μm) with a Zorbax eclipse plus C-18 guard column (4.6 mm×12.5 mm, 5 μm) [36]. The injection volume was 2 μL and the column temperature was 45°C. Separation was obtained by using a gradient of methanol/water 85:15 (eluent A) and iso-propanol (eluent B), programmed as follows: 90% A for 5 min, followed by a linear gradient to 90% B in 30 min, then 10 min at 90% B. Re-equilibration time for each analysis was 10 min. The chromatographic runs were performed at a flow

rate of 0.3 mL/min. The Jet Stream ESI operating condition for the positive mode were: drying gas (N_2, purity >98%): 350 °C at 10 L/min; capillary voltage 4.5 kV; nebulizer gas 35 psig; sheath gas (N_2, purity >98%): 375 °C at 11 L/min. High resolution mass spectra were acquired in the range 100–3200 m/z in positive mode. MS/MS spectra were acquired in the range 100-3200 m/z. The fragmentor was kept at 200 V, nozzle voltage 1000 V, skimmer 65 V, octapole RF 750 V. The voltage applied to the collision cell to induce CID for the MS/MS experiments was 50 V. The collision gas was N2 (purity 99.999%). Data were collected in Full Scan from 0 to 18 minutes for the best detection and quantification of derivatized fatty acids, and by auto MS/MS acquisition from 18 to 40 min for the best qualitative identification of DAGs and TAGs with a MS scan rate of 1.03 spectra/s and MS/MS scan rate of 1.05 spectra/s; only one precursor was acquired per cycle (relative threshold 0.010%), active exclusion after 3 spectra and 0.50 min (selection by abundance only).

The ESI operating conditions for the negative ionization mode were the same as those reported for the positive mode. For the MS/MS experiment the voltage applied to the collision cell to induce CID for the MS/MS experiments was 30 V. Data were collected by auto MS/MS acquisition with a MS scan rate of 1.03 spectra/s and MS/MS scan rate of 1.05 spectra/s. The mass axis was calibrated daily in positive and negative ionization modes using the Agilent tuning mix HP0321.

Triglycerides are named according to the following fatty acid abbreviations: Li: lignoceryl ($C_{24:0}$), Er: erucyl ($C_{22:1}$), B: behenyl ($C_{22:0}$), G: gadolenyl ($C_{20:1}$), A: arachidyl ($C_{20:0}$), Rn: ricinoleyl ($C_{18:1,OH}$), Ln: linolenyl ($C_{18:3}$), E: eleostearyl ($C_{18:3}$), L: linoleyl ($C_{18:2}$), O: oleyl ($C_{18:1}$), S: stearyl ($C_{18:0}$), Mr: margaryl ($C_{17:0}$),P: palmityl ($C_{16:0}$), P: palmitoleyl ($C_{16:1}$), M: myristyl ($C_{14:0}$), La: lauryl ($C_{12:1}$), Ca: capryl ($C_{10:0}$), Cl: caprilyl ($C_{8:0}$),Bu: butyryl($C_{4:0}$).

2.5. Data elaboration

MassHunter Workstation Software (B.04.00) was used to control the HPLC and the mass spectrometer, for data acquisition, and for data analysis. The relative abundances of the identified DAGs, MAGs and TAGs were calculated on the integrated peak areas under the $[M+Na]^+$ extracted ions (formula matching was set at 20 ppm tolerance with a limit extraction range of 1.5 min and an area filter of 500 counts. Formula having an isotopic pattern score lower than 25% were discarded) and normalized as reported in our previous works [37]. The fatty acid quantification was based on calibration curves reporting the integrated areas of the $[M+H]^+$ ions of the HQ derivatives as detected by the “Find by Formula” algorithm.

The calculation of the coefficients for the experimental design was operated with the open source software R Studio Version 1.0.136 and R version 3.3.2 (The R Foundation for Statistical Computing) with Chemometrics-with-R package. The maxima of the functions were calculated using Wolframalpha computational engine.

3. Results and discussion

3.1. Interpretation of mass spectra and tandem mass spectra

The mass spectra for the derivatized species were acquired and interpreted. The high resolution tandem mass spectrum of palmitic acid HQ derivative is shown in Figure 1 along with the interpretation of the observed ion fragments. All the analyzed fatty acid HQ derivatives followed the fragmentation pattern shown in Figure 1.

Table 1 reports the raw formulas and the exact masses of the fragment ions used for the development and validation of the analytical procedure, along with the formulas and the significant *m/z* values of the reagents and by-products of the derivatization reaction.

3.2. Optimization of the chromatographic separation

On the basis of a previously optimized gradient elution [37], the chromatographic conditions were improved in order to separate the derivatized fatty acids. The addition of water in the eluent mixture enabled the FAs to be successfully separated from C12 to C18, while the total analysis time changed from 40 to 45 minutes. The peak shape and the separation of MAGs and TAGs were improved by the addition of water in eluent A. Figure 2 shows the chromatogram acquired after the optimization, for the complete set of analytes (derivatized FAs, MAGs, DAGs and TAGs). The results prove that the analytes can be successfully separated and quantified in a single chromatographic run, and the FAs, MAGs, DAGs and TAGs profiled in less than 40 minutes.

The backpressure of the pump system ranges from 70 bar (at the beginning of the gradient) to 110 bar (at 90% *iso*-propanol), thus enabling us to work with a standard chromatographic pump, without using a high-pressure system. The new elution mixture led to an average decrease of 8.5% in the peak width for the triglycerides species with respect to those reported in our previous study [37].

3.3. Evaluation of the derivatization reaction kinetics and optimization of the derivatization step

We optimized the response for each analyte, defined as the integrated area normalized in terms of the actual concentration of the analyte in the individual solution. We took into account both temperature and reaction time. The temperatures tested were in the range 30-70 °C. Higher temperatures were not tested, since this would involve working above the boiling point of acetonitrile (82 °C).

The matrix used for the experimental design is provided in Table S1 along with the experimental results. The data show that the derivatization rate increased consistently with the reaction time. The response also increased from 30 °C to 50 °C, and decreased at 70 °C, suggesting degradation of the derivatized fatty acids. The repeatability (evaluated by calculating the CV% of three replicates) was satisfactory for each set of samples (lower than 15%), and did not show a specific trend when varying the temperature and reaction time.

Experimental data were used to calculate the coefficients b_0-b_5 of the function $R = b_0 + b_1t + B_2T + b_3tT + b_4t^2 + b_5T^2$ describing the fitting equation for the response for each analyzed specie depending on time (t) and temperature (T). Values are reported in Table S2. The coefficients and the best conditions, obtained by finding the maxima of the response function depending on time and temperature, differed for the five species investigated, ranging from 58 °C to 62 °C for the temperature, and from 5.5 to 6.2 hours for the reaction time. The 3D plots for the five functions are reported in Figure 3. In order to adopt reaction conditions that were optimal for all analytes, average time and temperature values were chosen: 60 °C for 6 hours. The reaction time, six hours, was above the reaction time range tested in our model. Thus, we used the calculated functions to predict the responses at 60 °C for six hours for each analyte. These are reported in Table S2 along with the experimental responses obtained performing six intra-day replicated experiments. The predicted and experimental values were compared using the *t*-test. At the 95% confidence level, the experimental results did not differ significantly from the theoretical values, proving the validity of the model in the optimal average conditions.

The stability of the derivatized species was also evaluated by injecting the same sample six times at 30 minutes intervals, and the intra-day precision was evaluated. No significant differences (N=3, 95% confidence level) were found for the peak areas with six subsequent injections (CV% < 4%), nor between the average response values of two triplicate analyses performed on different days (CV% < 15%). Thus, 60 °C for six hours were chosen as the optimal conditions, and 6 hours as the maximum time interval between sample preparation and injection.

3.4. Evaluation of the suitability of the derivatization reaction for the analysis of free fatty acids in the presence of glycerides

The suitability of the derivatization reaction for the identification of free fatty acids in complex lipid mixtures was assessed by evaluating the rate of transesterification of a solution of MAGs, DAGs and TAGs in the derivatization conditions. The results highlighted that the amounts of MAGs, DAGs and TAGs detected in the solution after derivatization was not statistically different from the results obtained without subjecting them to the derivatization reaction. Moreover, no fatty acid was found in the solution above the procedure detection limits. This indicates that the derivatization procedure does not lead to the hydrolysis or transesterification of acylglycerides.

3.5. Method validation

The method validation was performed using standard solutions. Precision, calibration curves, limits of detection, and limits of quantitation were reported in Table 2.

In detail, the limits of detection and quantitation were evaluated by analyzing analytical blanks, including the derivatization reagents and blank solutions. The limits of detection were in the range 0.2-2.0 ng/g, and the limits of quantitation were in the range 0.5-6.0 ng/g. The precision of the chromatographic method was evaluated by assessing intra-day and inter-day precision by analyzing a solution containing 20 ng/g of each standard, reporting the retention times and calculating the normalized areas.

The intra-day RSD on the retention times of the fatty acids HQ derivatives was lower than 0.3% and the intra-day RSD on the normalized area precision lower than 8%. The inter-day precision RSD on the retention times of the standards was lower than 0.8% and the inter-day precision RSD on the peak area repeatability is lower than 17%.

The calibration curves parameters were determined analyzing standard solutions at different concentration levels for each fatty acid: the selected concentrations were in the range 3-400 ng/g. The areas were plotted versus the concentration performing a least squares adjustment and obtaining r^2 coefficients for all the standards in the range 0.981-0.996, showing a good linearity in the investigated range (3-400 ng/g).

The new method has by better analytical performances with respect to the most sensitive methods presented in the literature, as reported in Table 3 [38-42]. The performances of the newly developed analytical method were compared to those that can be obtained by analytical procedures based on the solubilization of the sample, followed by HPLC/MS analysis of the lipid fraction in positive mode to characterize the glycerides, and subsequently by the HPLC/MS analysis of the lipid fraction in negative mode to characterize and quantify the fatty acids. To this aim standard solutions of fatty acids were analyzed by HPLC-ESI-Q-ToF in the negative mode, and limits of detection, limits of quantitation and dynamic linear ranges were calculated. Data presented in Table 3 show that the new proposed procedure displays better performances both in terms of detection limits (between two and three orders of magnitude) and linearity ranges (two orders of magnitude versus one).

Moreover, the method was validated by analyzing standard acyl glycerides. The limits of detection in the samples (LODs) for monopalmitin and dipalmitin were lower than 2.0 ng/g, while the limits of quantitation (LOQs) were lower than 6.9 ng/g. The triglycerides (trimyristin, tripalmitin, and tristearin) were characterized by LODs in the range 10-25 ng/g and LOQs in the range 30-80 ng/g.

The intra-day RSD on the retention times of the acyl glycerides was lower than 0.3%, and the intra-day RSD on the normalized area precision lower than 0.4%. The inter-day precision RSD on the retention times of the standards was lower than 0.6% and the inter-day precision RSD on the peak area repeatability lower than 0.7%.

The calibration curves were obtained using standard solutions at different concentration in the range 3.0×10^{1}-2.6×10^{4} ng/g. R^2 for all the calibration curves were higher than 0.995.

3.6. Characterization of the glyceride profiles of commercial oils and milks

The method was applied to the characterization of samples of commercial olive oils and milks, to prove its ability to profile free fatty acids, MAGs, DAGs and TAGs in a single chromatographic run, and to allow the quantitation of the free fatty acids in the same run. The re-

sults of the fatty acid quantitative analysis are reported in Table 4. The HPLC-MS chromatograms obtained for the extra virgin and sansa olive oils where characterized by a very similar lipid profile from a qualitative point of view, and are reported in Figure 4. The FFAs profiles of both oils were characterized by the presence of lauric, myristic, palmitic, oleic, linoleic, and stearic acid. Traces of linolenic acid were detected in the extra virgin olive oil only. From a quantitative point of view both oils were characterized by oleic acid as the main free fatty acid. The total amount of FAAs in the extra virgin olive oil was 1.0%, while in the sansa olive oil it was 0.1%, in agreement with the literature [43-47].

The acylglyceride profile for both olive oil samples are reported in Table 5 and, as for the FFAs profile, were in agreement with the literature [37, 48]. The relative abundances of the identified TAGs were calculated based on the areas of the peak of the $[M+Na]^+$ extracted ions and normalized as described in [37]. This approach is commonly used in the literature [43-45, 47, 49], and is based on the observation that in the adopted conditions the yield of the $[M+Na]^+$ is extremely reproducible, and can be thus used for semi-quantitative purposes [50-54]. Interestingly, by a semi-quantitative point of view, the diglycerides to triglycerides ratio was opposite for olive and sansa oil: the latter was characterized by the presence of higher amounts of diglycerides, probably due to the specific conditions of the extraction method used to produce this oil.

In order to explore the possibility to use this new analytical approach to evaluate the adulteration of milk, the method was applied for the characterization of acyl glycerides and fatty acids of a sample of cow whole milk and a sample of a commercial infant milk formula produced with a mixture of cow milk and different vegetable oils. Phospholipids were not investigated in this analysis, as they were previously precipitated together with proteins in acetone [34, 35]. The chromatographic profiles are reported in Figure 5 and Figure S1.

Both milk samples showed the presence of fatty acids characterized by chain lengths ranging from 6 to 18 carbon atoms (Figure S1 supplementary), with palmitic acid as the most abundant. Traces of linoleic and linolenic acid were detected only in the infant formula, most likely deriving from the vegetable oils used in the formulation. Quantitative analysis of fatty acids was performed, and results are reported in Table 4: both milks were characterized by <0.1% of FFAs.

The triglycerides identified in the chromatographic profile of the milk samples are reported in Table S.3 with the relative mass spectra interpretation. The profile obtained for whole cow milk was in agreement with those reported in literature, with the presence of several triglycerides containing short chain fatty acids as acyl substituents (butyric, caproic, caprylic, and capric acids as the most abundant) [55]. The infant formula was characterized by the presence of the same triglycerides detected in cow milk as well as several other triglycerides ascribable to vegetable oils. In detail, the chromatogram of the infant formula extract was characterized by the presence of trilaurin (LaLaLa) [56] ascribable to coconut oil, LnLnL, LnLL, PLnP, LLL, ALL, ALO, AOO, BOO, BLO, and BLL ascribable to sunflower oil and rapeseed oil [57]. The results agree with the information on the label of the infant formula milk.

For both types of samples, olive oil and milk, the detection of the main components (DAGs and TAGs) and of the minor species (FFAs) was accomplished in a single run, thus proving the suitability of the proposed method in terms of wide range of applicability.

4. Conclusions

We developed a sensitive HPCL-ESI-Q-ToF method for the simultaneous identification, separation and quantification of free fatty acids and acylglycerides in lipid samples. The procedure is well suited for characterizing samples containing minor amounts of FFAs, such as plant oils, or for the detection of frauds such as the addition of low quality oils to extra virgin olive oil. Moreover, oils and fatty acids can be added as additives or even adulterants in infant milk formula.

FFAs were analyzed, in a single chromatographic run together with glycerides, after derivatization with 2-hydrazinoquinoline, which is a low-cost analytical approach. The conditions used for the derivatization of fatty acids were optimized using a full factorial experimental design and the approach was fully validated.

The method presents a good linearity range, very high reproducibility and low detection and quantification limits (lower than 1.9 ng/g and 6.4 ng/g in the sample, respectively). The performances of the procedures in terms of detection limits and dynamic range were compared to those achieved by the detection in negative mode of the underivatized FFAs and proved superior of two orders of magnitude. In addition, the new chromatographic conditions used for the separation of the species enabled us to improve the chromatographic performances, with an increase of 8.5 % in the number of theoretical plates for the triglycerides species with respect to those reported in our previous studies [37].

The analytical approach proved to be sensitive, highly selective and robust. It was applied to characterize an extra virgin olive oil and a sansa olive oil (i.e. low quality), showing that the procedure facilitates their differentiation from both a quantitative and qualitative point of view. The method was also tested for the characterization of whole cow's milk, as well as of an infant milk formula containing vegetable oils, after precipitation of proteins and phospholipids in acetone. The method allowed the qualitative and quantitative evaluation of the FFA and acylglycerides composition in lipid samples, showing to be a valuable tool to support studies on the chemical composition of complex matrices. By featuring implementations specific to the chemistry of the materials investigated, this method is in fact very promising for a variety of applications, from the investigation of adulterations in commercial products, to industrial quality control, as well as the characterization of lipids in botanical extracts, food industry and oil paints in cultural heritage.

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Table 1- Raw formulas and exact masses of the analyzed ions for each species used in the optimization of the analytical procedure, along with the detected product ions. The formulas and m/z values for the derivatization reaction reagents and by-products are also reported.

	Common name	Raw formula	Formula of the derivatized compound	Retention times (min)	m/z	Product ions (%)
PyS	2-pyridylthiol	C_5H_5NS	-	1.3	112.022, $[M+H]^+$	-
HQ	2-hydrazino quinoline	$C_9H_9N_3$	-	1.4	160.087, $[M+H]^+$	116.051 (100), 142.031 (32), 160.087 (1.5)
monoP	monopalmitin	$C_{19}H_{38}O_4$	-	1.6	353.267, $[M+Na]^+$	108.011 (10), 183.043 (100) 353.267 (5)
DPDS	2,2'-dipyridyl disulfide	$C_{10}H_8N_2S_2$	-	2.0	221.020, $[M+H]^+$	110.006 (6), 111.014 (100), 187.032 (23)
C12	lauric acid	$C_{12}H_{24}O_2$	$C_{21}H_{31}N_3O$	3.4	342.254, $[M+H]^+$	116.049 (74), 128.049 (6), 143.060 (100), 160.086 (3)
			-	2.1	199.173, $[M-H]^-$	-
TPP	triphenyl phosphine	$C_{18}H_{15}P$	-	4.1	263.098, $[M+H]^+$	152.063 (22), 183.087 (100), 263.098 (3)
PP	dipalmitin		-	4.7	591.496, $[M+Na]^+$	109.008, (100) 313.263 (80), 591.496 (20)
C14	myristic acid	$C_{14}H_{28}O_2$	$C_{23}H_{35}N_3O$	5.5	370.285, $[M+H]^+$	116.049 (48), 143.060 (100), 160.08 (5)
			-	3.2	227.204, $[M-H]^-$	-
TPP-Ox	triphenyl phosphine oxide	$C_{18}H_{15}OP$	-	5.8	279.093, $[M+H]^+$	128.062 (65), 133.020 (43), 141.069 (16), 154.077 (42), 171.035 (93), 183.035 (59), 201.046 (100)
C16	palmitic acid	$C_{16}H_{32}O_2$	$C_{25}H_{39}N_3O$	9.4	398.316, $[M+H]^+$	116.049 (38), 128.049 (3), 143.060 (100), 160.086 (9)
			-	5.3	255.235, $[M-H]^-$	-
C18:1	oleic acid	$C_{18}H_{34}O_2$	$C_{27}H_{41}N_3O$	10.9	424.332, $[M+H]^+$	116.049 (28), 143.060 (100), 160.087 (12)
			-	6.2	281.253, $[M-H]^-$	
C18	stearic acid	$C_{18}H_{36}O_2$	$C_{27}H_{43}N_3O$	13.9	426.347, $[M+H]^+$	116.050 (27), 143.060 (100), 160.087 (13)
			-	9.9	283.269, $[M-H]^-$	-
MMM	trimyristin	$C_{45}H_{86}O_6$	-	13.6	745.625, $[M+Na]^+$	109.0988 (20), 132.114(12), 137.129 (6), 211.201 (14), 495.4352 (100), 517.417 (20), 745.625 (30)
PPP	tripalmitin	$C_{51}H_{98}O_6$	-	19.5	829.716, $[M+Na]^+$	109.098 (20), 132.114(12), 137.129 (6), 239.231 (10), 551.493 (100), 573.478 (40), 829.716 (50)
SSS	tristearin	$C_{57}H_{110}O_6$	-	24.03	913.823, $[M+Na]^+$	109.098 (9), 132.114(6), 137.129 (3), 267.626 (15), 607.555 (70), 629.537 (50), 913.823 (100)

Table 2 - Parameters calculated in the method validation for both the positive and negative detection modes: limit of detection (LOD), limit of quantitation (LOQ), Linear dynamic range.

Acquisition mode		Figures of merit (ng/g)				
		C12	**C14**	**C16**	**C18:1**	**C18**
Positive. (2-HQ derivates)	**LOD**	9.0×10^{-1}	2.0×10^{-1}	1.8	1.1	1.9
	LOQ	3.0	5.0	6.0	3.8	6.4
	Dynamic Range	$3.0 - 4.0 \times 10^{2}$	$5.0 - 4.0 \times 10^{2}$	$6.0 - 4.0 \times 10^{2}$	$4.0 - 4.0 \times 10^{2}$	$7.0 - 4.0 \times 10^{2}$
Negative	**LOD**	1.0×10^{2}	1.1×10^{2}	1.4×10^{2}	1.2×10^{2}	1.4×10^{2}
	LOQ	3.2×10^{2}	3.8×10^{2}	4.5×10^{2}	3.9×10^{2}	4.6×10^{2}
	Dynamic Range	$3.0 \times 10^{2} - 2 \times 10^{3}$	$4.0 \times 10^{2} - 1.0 \times 10^{3}$	$5.0 \times 10^{2} - 2.0 \times 10^{3}$	$4.0 \times 10^{2} - 2.0 \times 10^{3}$	$5.0 \times 10^{2} - 2.0 \times 10^{3}$

Table 3 – Comparison between the performances of the new procedure with the most sensitive methods recently proposed in the literature.

Column	*Eluents*	*Derivatization reagents*	*Injection volume*	*Detector*	*LOD*	*LOQ*	*Ref.*
Poroshell 120 EC-C18 (3.0 x 50 mm, 2.7 µm, Agilent Technologies, USA)	*iso*-propanol methanol water	2-HQ	2.0 µL	ESI(+)-Q-ToF	0.2-1.9 ng/g (0.3- 3.0 pg; 1.0-11 fmol)	3.0-6.4 ng/g (4.7-10 pg; 4.0-3.6 fmol)	*This paper*
Zorbax SB-C8 (2.1 x 100 mm, 1.8 µm Agilent Technologies)	acetonitrile water	DMMP	10 µL	ESI(+)-QqQ	5.0-15 pmol	-	[37]
Ascentis Express C18, (2.1 x 150 mm, 2.7 µm, Sigma-Aldrich, USA)	acetonitrile water modifier: acetic acid	AMMP	-	ESI(+)-QqQ	-	10 pg	[38]
Cadenza CD-C18 (2.0 x 150 mm, 3.0 µm, Imtakt, Japan)	acetonitrile water formic acid	DAABD-AP DAABD-AB	-	ESI(+)-Q-ToF	0.1-0.12 pmol	-	[39]
YMC-Pack Pro C18 RS column (2.0 x 150 mm, 2.7 µm, YMC, Japan)	methanol water modifier: ammonium formate	1-DAPAP	15 µL	ESI(+)-QqQ	1.0-2.2 fmol	-	[40]
Nucleoshell Gravity C18 (2.1 mm × 100 mm, 1.9 µm, Macherey-Nagel, Germany)	acetonitrile water modifier: acetic acid	AMMP PFB	10 µL	APCI(+)-QqQ	3.0 pg-0.03 ng	-	[41]
HiChrom RPB column (2.1 mm × 250 mm, 5 µm, HiChrom Ltd, UK)	water methanol modifier: formic acid	DAABD-AE	-	ESI(+)-QqQ	0.001-0.010 µg/g	0.01-0.1 µg/g	[42]

Abbreviation list: 1-DAPAP: (S)-1-(4-Dimethylaminophenylcarbonyl)-3-aminopyrrolidine; DAABD-AP: 4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminopentylamino)-2,1,3-benzoxadiazole; DAABD-AB: 4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminobutylamino)-2,1,3-benzoxadiazole; AMMP: N-(4-aminomethylphenyl)pyridinium; DMMP: 2, 4-dimethoxy-6-piperazin-1-yl pyrimidine; AMMP:(N-(4-aminomethylphenyl)pyridinium chloride); PFB: pentafluorobenzyl bromide; DAABD-AE: 4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2- aminoethylamino)-2,1,3-benzoxadiazole

Table 4 – Quantitative analysis results in terms of μg per gram of sample, obtained for the commercial oil samples and milk samples. The RSD% was lower than 10% for all the samples analyzed. Σ_{FFA} (%): total percentage of free monocarboxylic fatty acids in the sample.

	Extra virgin olive oil	Sansa olive oil	Cow milk	Infant milk
Lauric acid C12 (μg/g)	2.3×10^{1}	7.4	2.5	2.9
Myristic acid C14 (μg/g)	4.0×10^{1}	1.3×10^{1}	6.4	6.3
Palmitic acid C16 (μg/g)	1.5×10^{3}	1.7×10^{2}	4.0×10^{1}	2.3×10^{1}
Oleic acid C18:1 (μg/g)	8.4×10^{3}	9.4×10^{2}	1.6×10^{1}	1.1×10^{1}
Stearic acid C18 (μg/g)	3.0×10^{2}	2.9×10^{1}	4.2×10^{-2}	8.5×10^{-2}
Σ_{FFA} (%)	1.0	0.1	<0.1	<0.1

Table 5 – Relative abundance (%) of the main identified triglycerides in the commercial oil samples. The relative abundances of the identified TAGs were calculated on the integrated peak areas in the $[M+Na]^+$ extracted ions (Formula matching was set at 20 ppm tolerance with a limit extraction range of 1.5 min and an area filter of 500 counts) and normalized as described in [37]. The triglycerides were identified by high resolution mass spectrometry. The tandem mass spectra interpretation is reported in [37].

TAGs	m/z $[M+Na]^+$	Extra virgin oil	Sansa olive oil
LLL	*901.718*	3.3	2.6
LLP	*877.724*	8.6	8.3
LLO	*903.744*	6.0	10.0
PLP	*853.753*	3.6	5.1
LOP	*879.731*	9.8	14.0
OOL	*905.760*	11.1	15.2
POP	*855.755*	2.9	4.8
OOP	*881.770*	5.5	8.3
PPS	*857.762*	0.0	0.0
OOO	*907.791*	36.3	15.4
OSP	*883.788*	2.8	4.0
OOS	*909.830*	8.0	10.4
PSS	*885.781*	0.0	0.0
OSS	*911.808*	2.0	1.9

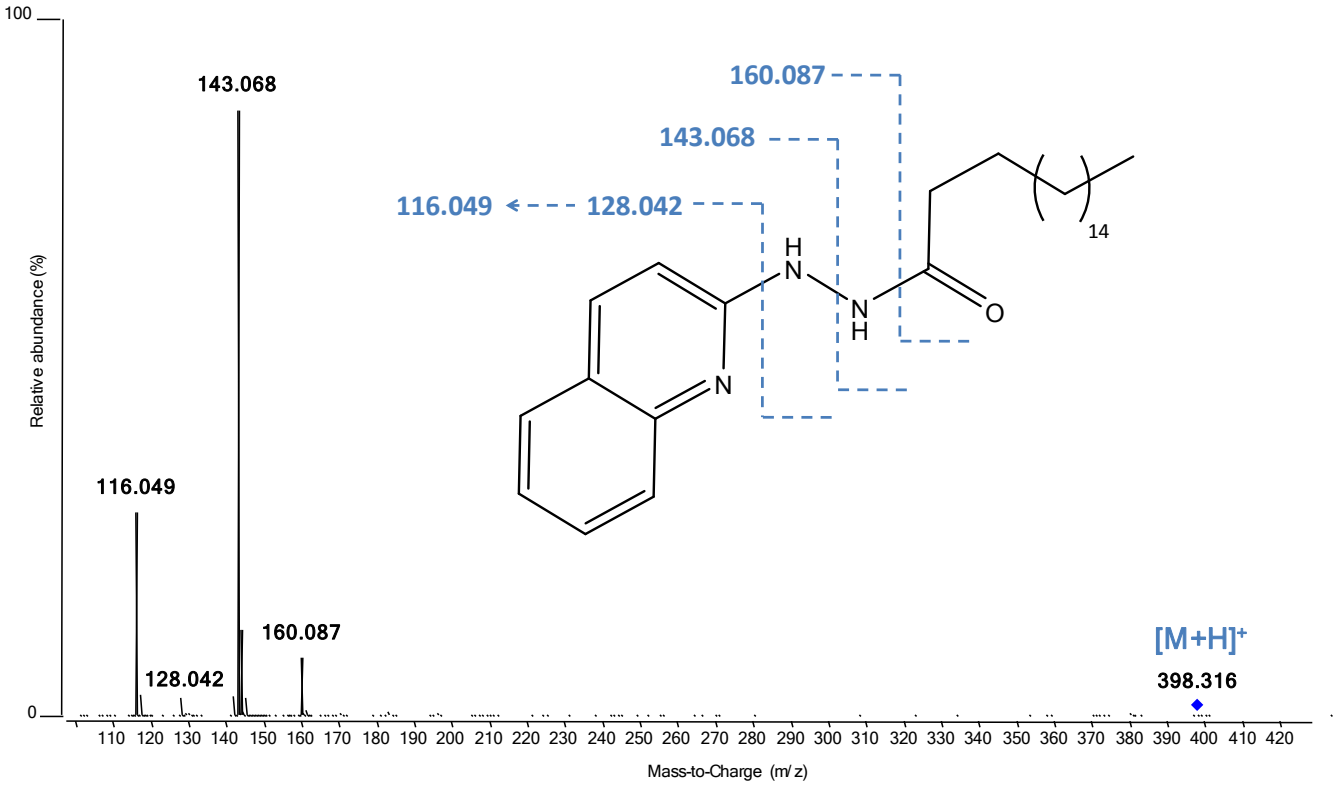

Figure 1 –CID mass spectra obtained for derivatized palmitic acid.

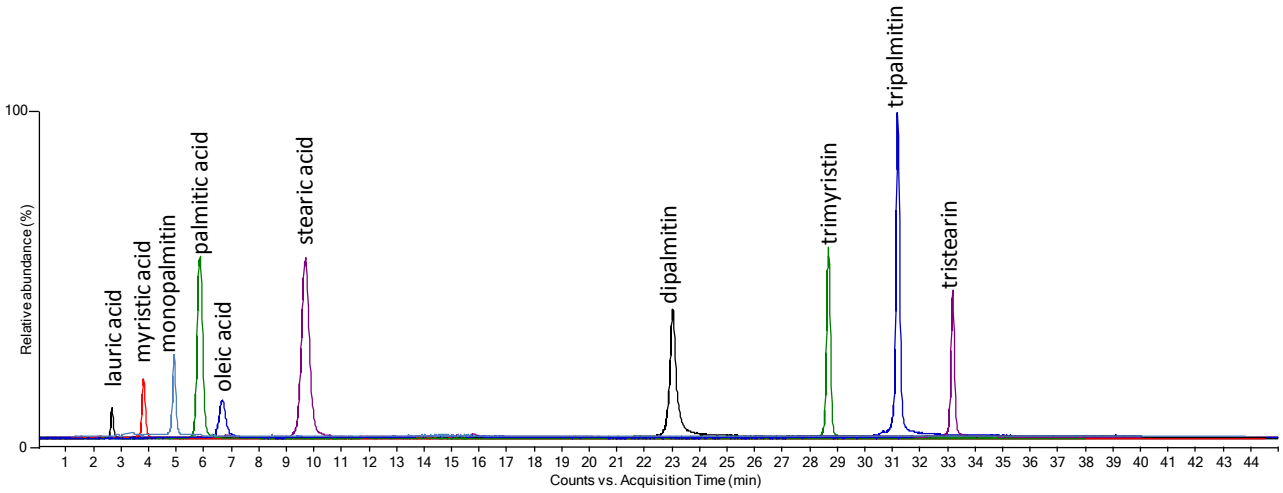

Figure 2 - Extract ion chromatograms of the standard derivatized fatty acids together with monopalmitin, dipalmitin, trimyristin, tripalmitin, and tristearin.

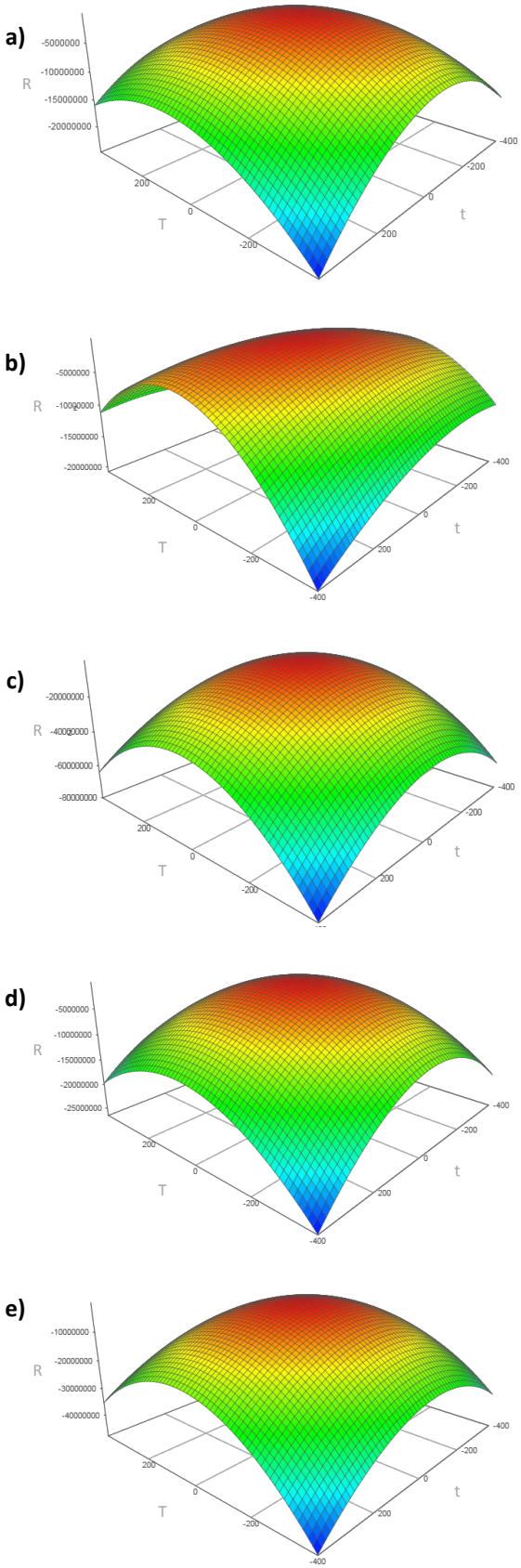

Figure 3 – 3D plot obtained for the Response in function of temperature (T) and time (t) of lauric (a), myristic (b), palmitic (c), oleic (d), and stearic (e) acids.

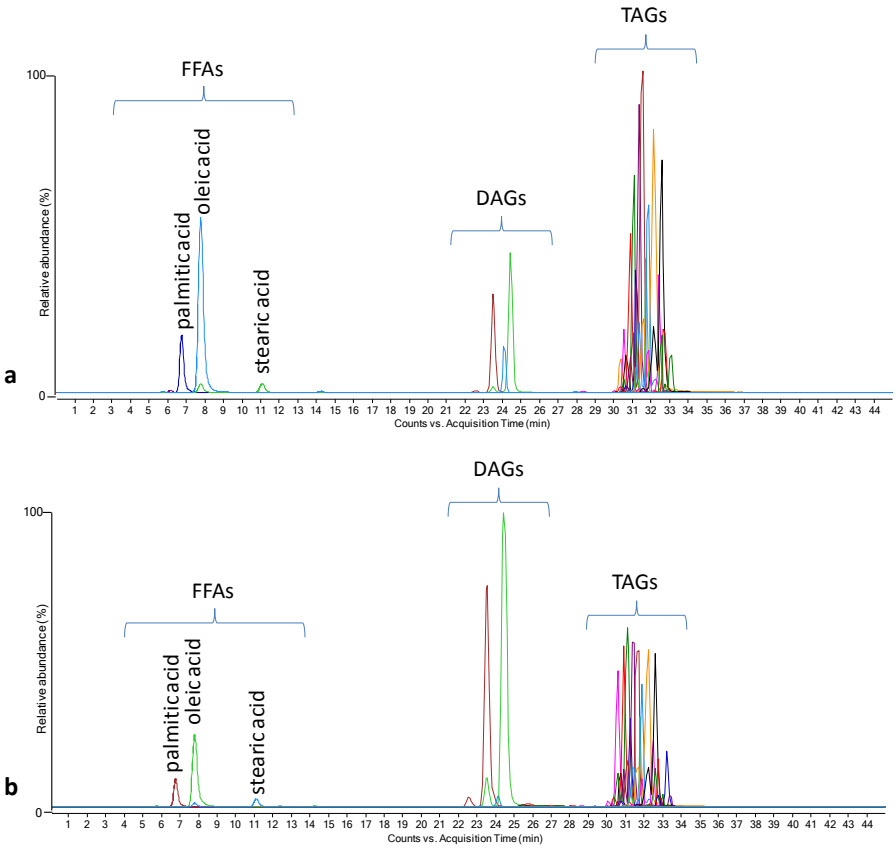

Figure 4 – HPLC-ESI-Q-ToF extract ion chromatograms of extra virgin olive oil (a) and sansa olive oil (b).

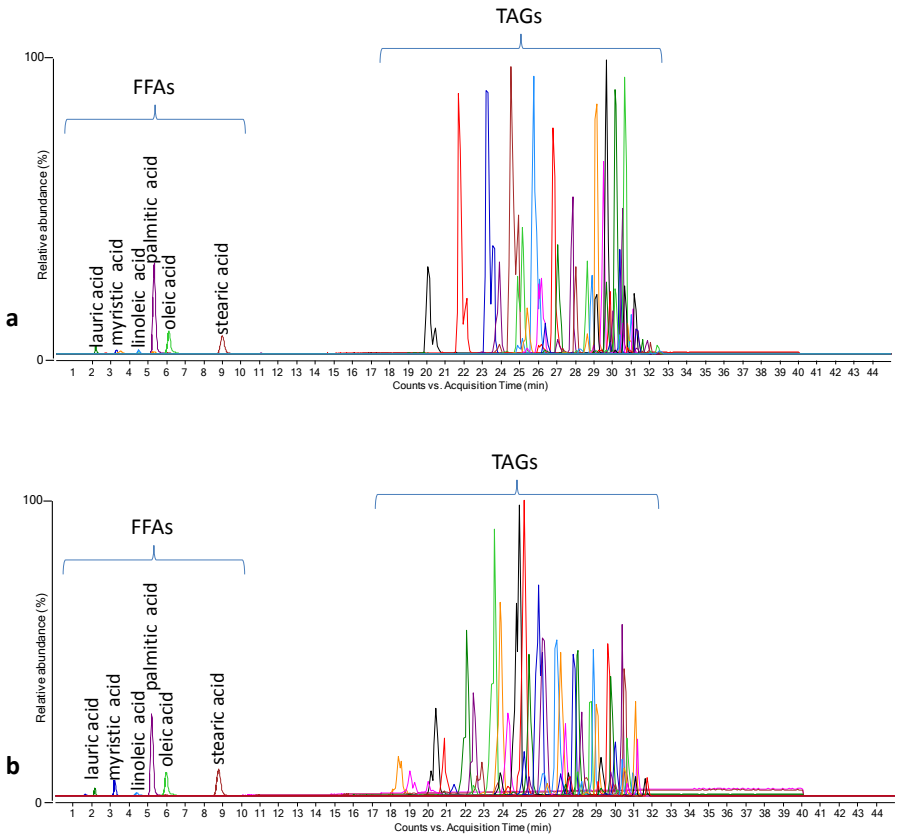

Figure 5 – HPLC-ESI-Q-ToF extract ion chromatograms (EICs) of whole cow milk (a) and infant milk (b). The EICs of short chain fatty acids detected in infant milk are shown in detail in Figure S1.