Determination of the palm oil addition in food

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Palm oil (PO) is the most widely consumed vegetable oil on the planet. PO has a high percent of saturated fatty acids that are not favourable to the health. From December 2014, all food items sold in Europe must list palm oil in their ingredients if it is in the product. However, many food items are declared as PO free, and in the actual regulation there are no official methods to verify this statement. Therefore, there is a strong need to develop analytical methods for this purpose.
In this work, screening methods with fast DART-MS analysis and confirmatory methods for the determination of PO in food were defined. DART-MS analysis with ion trap or high resolution mass spectrometry can be used as screening method, meanwhile the determination of both tocotrienols and triacylglycerols with HPLC can be used as confirmation method. PO addition was detectable up to 1%.

Keywords: food frauds, lipid analysis, palm oil.

1. INTRODUCTION

PO is the most widely consumed vegetable oil on the planet, and it is present in about half of all packaged products sold in the supermarket varying from bakery products, pizza dough and pasta to detergents and lipsticks. The demand for PO has increased steeply in last decades as a substitute of animal fat and as a low trans vegetable fat alternative to hydrogenated vegetable oils. It is produced in tropical areas and its rapid expansion threatens some of the planet's most important and sensitive habitats. Forest conversion to oil palm plantation continues to increase rapidly. Most PO plantations are located in Southeast Asia, but the forest conversion to oil palm plantation expansion will occur in Africa too. PO has a high percent of saturated fatty acids that are not favourable to health and recently the problems concerning food safety have evidenced the dangerous content of 3-MCPD in the refined product [1]. The consciousness of these facts has led the consumers to request food with no palm oil as ingredient. Starting from December 2014, all food products containing PO sold in Europe must state it in the ingredient list [2].
Many food items on the italian market are now advertised as PO free. 45% of consumers don't believe 'free from' claims [3]. Due to its low price, the rheological and stability properties, PO is a common ingredient in food and without an analytical method to detect its presence, it is possible to use it without a label declaration. For this reason, it is important to have reliable and easy analytical methods to prevent this food fraud. There are many articles where PO is characterised as fatty acids composition, tocopherols and tocotrienols [4, 5, 6, 7, 8], triacylglycerols (TAGs) [9] but there are no validated methods to verify its presence in food.

PO is composed of triglycerides (triacylglycerols, TAGs) (>90%), diglycerides (diacylglycerols, DAGs) (5%), monoglycerides (monoacylglycerols, MAGs) (1%), tocols (600-1000 mg/kg) subdivided in tocotrienols (76%) and tocopherols (24%), sterols (250-700 mg/kg) [10, 11]. Among fatty acids PO is particularly rich in palmitic acid (39,3-47,5%). Considering this composition, it would be possible to differentiate PO from other vegetable oils. As far as we know only few analytical methods have been proposed to verify the presence of PO in food. Due to the difficulties to discern mixtures of different type of vegetable oils they are applied to olive and PO blends [12] or in milk samples [13] and not in processed food. This last method utilizes magnetic dispersive solid-phase extraction, a technique with the disadvantage that the synthesis and pre-treatment processes of these magnetic sorbents are very time consuming and may involve toxic reagents. Several methods have been developed for the verification of olive oil frauds and sophistication using fatty acids composition, triglycerides, phytosterols [14, 15, 16, 17, 18] as well as PO composition was investigated for geographical origin [8, 9] and adulteration [19, 20, 21, 22]. Nevertheless, the PO usage in food became a relevant question for consumers, there are only few works on it. In recent works hydrophobic magnetic nanoparticles were used for the detection of PO in milk [13] and fatty acid composition was used to quantify the percentage of PO in olive oil [12]. The aim of this work was, in a preliminary phase, to verify the possibility to detect PO addition in food using different methods comprising classical analysis with chromatographic separations (GC and HPLC) and the fast Direct Analysis in Real Time approach (DART-MS).

2. EXPERIMENTAL PART

2.1. REAGENTS AND STANDARDS

All the reagents used were of ACS quality grade. FAME mix (37 components 200-600 µg/mL in methylene chloride, Supelco), cis-vaccenic acid methyl ester (C18 11:1 11c), trans-vaccenic acid methyl ester (C18:1 11t), docosatetraenoic acid methyl ester (C22:4 omega 6), docosapentaenoic acid methyl ester (C24:5 omega 3), stearidonic acid methyl ester (C18:4 omega 3), Alfa, beta, gamma, delta tocopherols (purity 99%) were purchased from Merck - Sigma – Aldrich (Darmstadt, Germany). Ethyl acetate reagent grade was purchased by VWR International, Ltd. (Poole, England). SPE silica column 1g/6 mL were purchased from Waters – Italy. Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

2.2 SAMPLES AND SAMPLE PREPARATION

A set of 32 food items (processed food with and with-

out PO addition declared in the label) were chosen from the market including confectionary and bakery products, infant formulas, spreadable creams, snacks and oils as shown in Table I. After extraction the resulting fat was used for all the determination.

Fat extraction: 40 g of product were grinded avoiding heating, extracted using 200 mL of petroleum ether using 30 min ultrasonic bath followed by 60 min of shaker at room temperature and, at the end, filtered on paper and evaporated under reduced pressure to dryness. To assure the effectiveness of palm oil extraction, addition tests (n = 6) were conducted on representative food (crackers, biscuits, snacks, breadsticks), adding 1 g (2.5%) of PO before extraction and measuring the weight difference with and without addition: the recovery were over 85% for all the tests (90.1% mean; 6.4% standard deviation).

For triglycerides and tocopherols characterization vegetable samples well known for their composition were extracted and analysed for the peaks' identification (e.g. palm oil, palm olein, olive oil for triglycerides identification and a blend of wheat germ oil/soya bean oil/palm oil 15/38/48 w/w/w for tocotrienols and tocopherols identification).

2.3 GC-FID ANALYSIS CONDITIONS (FATTY ACIDS DETERMINATION)

0.1 g of oil/fat was dissolved in 2 mL of heptane, 2 mL of KOH 2M in methanol were added and, after shaking for 30 sec, the clear upper phase was injected in GC-FD system. The analysis was performed using an Agilent 6890N gas chromatograph coupled with a flame ionization detector (GC-FID). The GC-FID conditions were as follow: highly polar bis-cyanopropyl 100 m × 0.25 mm × 0.20 µm film thickness column Supelco SP-2560; injection volume 2 µL with a split 1:200; carrier gas He at flow rate of 40 mL/min; injection temperature 250°C, oven temperature140°C for 4 min, 240°C at 4°C/min, 240°C for 15 min FID temperature 260°C.

2.4 HPLC-FLUORESCENCE DETECTOR ANALYSIS CONDITIONS (TOCOPHEROLS AND TOCOTRIENOLS DETERMINATION)

A sample of extracted fats (about 0.4/0.5 g) was

Table I - Food items used

Food item	Number	Food item	Number
Palm oil	3	Rusks	1
Coconut-palm kernel oil mixture	1	Breadsticks	1
Biscuits	13	Infant formula	2
Potato snacks	3	Peanuts-palm oil mixture	1
Crackers	6	Croissant	1

weighted in a 10 mL graduated flask, solved and filled to volume with isopropanol. The solution was filtered on nylon membrane syringe filter 0,45 μm, 4 mm before to be injected in the HPLC system. A reverse phase column (Cosmosil π NAP 15 cm × 4.6 mm, 5 μm, 120°A, CPS – Italy) refrigerated at 20°C was used.

A HPLC (Spectra System – Italy) system equipped with a fluorescence detector FL3000, with a ternary gradient pump P4000 and a solvent degasser SCM1000 was utilized. The fluorimetric detector was programmed with an excitation wavelength of 294 nm and an emission wavelength of 330 nm.

Mobile phase constituted as initial composition of water 0.2 % H_3PO_4/methanol 16/84 at a flow rate of 1.00 mL/min, isocratic for 28 minutes was used. Then the column was washed with acetonitrile for 15 minutes at a flow rate of 1.0 mL/min. The reconditioning time was 15 minutes.

An external standard solution of tocopherols was prepared in isopropanol at a concentration level of 0.1 mg/20 mL in isopropanol and 20 μL were injected in the HPLC system.

Two independent determinations were conducted for each analysed sample. A blank control constituted by the injection of 20 μL of isopropanol was analysed before each batch of samples. The response factor of the four tocopherols standards was recorded and used for the calculation of tocopherols/tocotrienols forms.

2.5 HPLC-RI ANALYSIS CONDITIONS (TAG'S DETERMINATION)

The sample, 120 mg/0.5 mL in hexane, was purified on a silica SPE column eluting the purified triacylglycerol fraction with a solution of petroleum ether/ethyl ether 87/13 v/v. 10 μL were injected in the HPLC-RI system. The triacylglycerols profile was recorded utilising the same analytical conditions of ECN42 method [23] using propionitrile at a flow rate of 0.5 mL/min as mobile phase, a HPLC system equipped with a refractive index (Spectra-System – Italy). The column was thermostated at 25°C.

2.6 DART-ION TRAP ANALYSIS CONDITIONS

A Deca XP Plus (Thermo Fisher Scientific, Fair Lawn, NJ) mass spectrometer, polarity positive, mass range 300-830 amu, nano spray ionization (NSI) 0.310, collision energy 40%, precursor ion 824 m/z, product ions 550, 524 m/z was used.

The DART conditions were: temperature 350°C, grid voltage 250 V, gas helium, polarity positive, speed 0.3 mm/s. To enhance the ionization a 2 mL vial containing an aqueous solution of ammonia (25% w/w) was placed near the DART gun exit. 1 mL of oil/fat was extracted with 10 mL of pure ethyl acetate and placed in ultrasound bath at room temperature for 10

min. Finally, 5 μL of the solutions were introduced into the instrument using Dip-it tips.

2.7 DART-HRMS ANALYSIS CONDITIONS

An Exactive Plus (Thermo Fisher Scientific, Waltham, MA,) mass spectrometer equipped with a DART-SVP with DART SVP 100 ion source (IonSense, Saugus, MA) was used.

Mass conditions were: polarity positive, mass range 75-1250 m/z, resolution 70.000 FWHM, analysis time 0.66 min, AGC target 3e6 capillary 250°C, S-lens RF 75, CID 0 eV, T_{inj} max 10 ms

1 mL of oil was extracted with 10 mL of pure ethyl acetate and placed in ultrasound bath at room temperature for 10 min. Finally, 5 μL of the solution was introduced into the instrument using Dip-it tips.

DART software (IonSense) controlled DART functions, the gas temperatures and the velocity of the 12-Dip-it auto-sampling (IonSense) movement. A vapor interface (IonSense) was used to direct ions from the ion source to the capillary inlet of the MS. The distance between the exit of the DART gun and the ceramic transfer tube of the vapor interface was 2.4 cm. Other optimised DART settings were the grid voltage: 250V, inlet gas temperature: 350°C, sample speed: 0.5 mm/sec with a single time analysis of 0.66 min. Setting of the system parameters for mass spectrometric detection were: S-lens RF level: 75, capillary temperature: 250°C; maximum injection time: 10 ms.

3. RESULTS AND DISCUSSION

To simulate the adulteration and to establish a limit of detection for the addition, all the methodologies has been also applied in a mixture of sunflower or rapeseed oil with palm oil at the percentages of 1, 2, 5, 10, and 50%. Sunflower and rapeseed oils were chosen as the most used oils in food composition.

To detect the PO addition there was evaluated the presence of its characteristic components, like some fatty acids, TAG's and tocotrienols.

3.1 FATTY ACIDS RESULTS

The different composition of palm oil respect to the other oils could lead to detectable differences. PO has a higher percentage of saturated FA, especially palmitic acid and a lower percentage of long chain and unsaturated FA. Some works concern PO adulteration with other oils or fat using FA composition were published [20, 22] but none in processed food. FA composition in PO varies with the origin and, furthermore, the fat composition of complex matrices such as confectionery and bakery products is enriched by the presence of mono and diglycerides of FA as emulsifier and this fact complicates the discrim-

ination. 32 samples were analysed (see *2.2. Sample and sample preparation*). Analysing the rough data, it was impossible to distinguish PO presence and, consequently, a statistical approach was necessary. PCA was performed on the FA data of 32 samples and 42 variables. In our experiments, PCA scores plot (Fig. 1A) showed a rough distinction between products with and without PO. Palmitic, oleic, lauric and linoleic acids have the largest loading (Fig. 1B). The PO containing products was grouped along with three samples of PO and palm kernel oil; two samples (breadsticks with PO and extra-virgin oil, infant formula with PO, sunflower, and rapeseed oil) were inside the without PO group. Peanut butter with PO addition was confused in the group without PO too. A possible interpretation of this results is that high percentage of another type of oil other than palm is enough to remove the sample from PO group making this analysis scarcely reliable. Also, mixtures of sunflower oil or rapeseed oil with concentration of PA 10% were scarcely detectable. Giving false positive this analysis is not usable as screening analysis.

3.2 TOCOTRIENOLS RESULTS

This analysis was performed with HPLC with fluorescence detector on a naphthalene bonded stationary phase column where it was possible to clearly separate the different tocopherols and tocotrienols peaks. In crude palm oil total tocopherols content ranges from 700 to 1000 mg/kg [24], with tocotrienols that are about 75% of the total [6], with a prevailing form in gamma-tocopherol. Tocopherols and tocotrienols were determined in PO [7] and this oil is considered a good source of tocotrienols such as grapeseed oil and wheat germ oil, corn, rice. Its presence in food could reveal addition. This analysis alone does not resolve the problem, totally giving false positive results and it must be performed in parallel with the triacylglycerol

profile determination. The limit of detection calculated analysing the different blends prepared, and based on signal to noise ratio (3/1) was 1% palm oil addition on sunflower and rapeseed oil. Figure 2 shows the chromatogram of a sunflower (A) and rapeseed (B) oil without and with 5% PO addition.

3.3 HPLC-RI TAG's RESULTS

The triglycerides analysis was conducted on an octadecyl HPLC column using propionitrile as an eluent and a refractive index detector. The addition of palm oil was observed with the presence of PPP peak and the increase of PPO peak, characteristic of palm oil. PPP is not specific of palm oil, coco and palm kernel oils contain it too, but the first one has a typical taste, it is difficult to use in food production and the latter is not cheap and is mainly used in the cosmetic field. The limit of detection was 1% of PO addition (signal to noise ratio 3/1). This analysis, along with tocotrienols one, could be considered as a confirmatory method and they were used to define the presence/absence in the set of 32 food samples that were analysed with the other techniques too (GC-FID, DART-HRMS, DART-ion trap). Figure 3 shows the chromatogram of a rapeseed (A) and sunflower (B) oil with and without 2% PO addition.

3.4 TAG's DART/HRMS RESULTS

Since the introduction of the direct analysis in real time (DART) [25], this technique was used for the analysis of food [26], and it was demonstrated to be a suitable technique for fat and oils analysis [27, 28], milk and milk based foods [29] and for general food-quality and safety analysis [26, 30, 31, 32].
In this experiment DART was coupled with a high-resolution mass spectrometer exploring the possibility to discriminate the "pseudo isobaric" molecules.
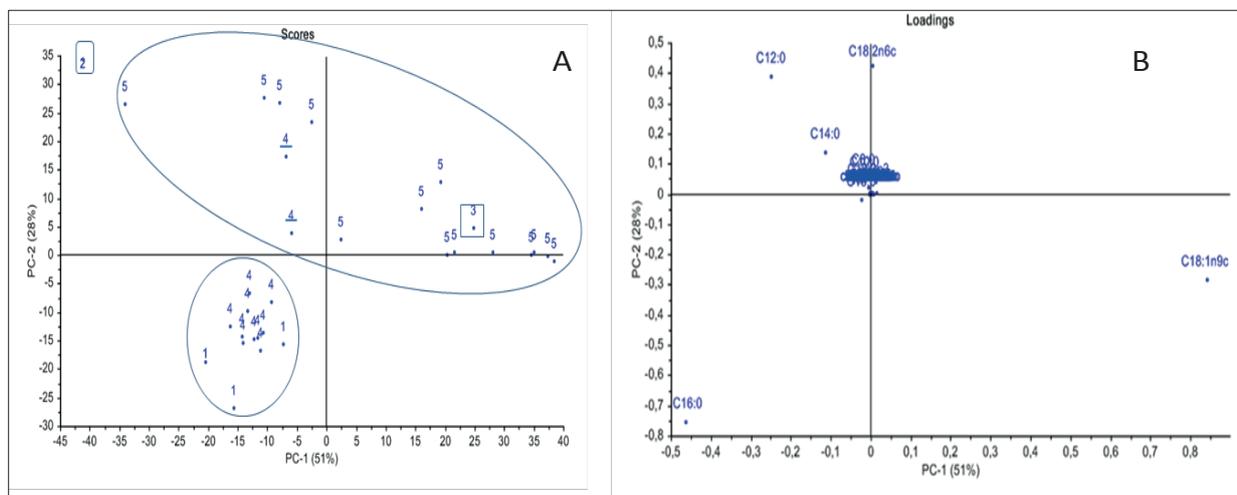

Figure 1 - (A) Scores plot of FA data: 1 PO, 2 coco/palm kernel mix, 3 peanut butter with PO;4 products with PO; 5 products without PO. (B) loading plot of FA data.
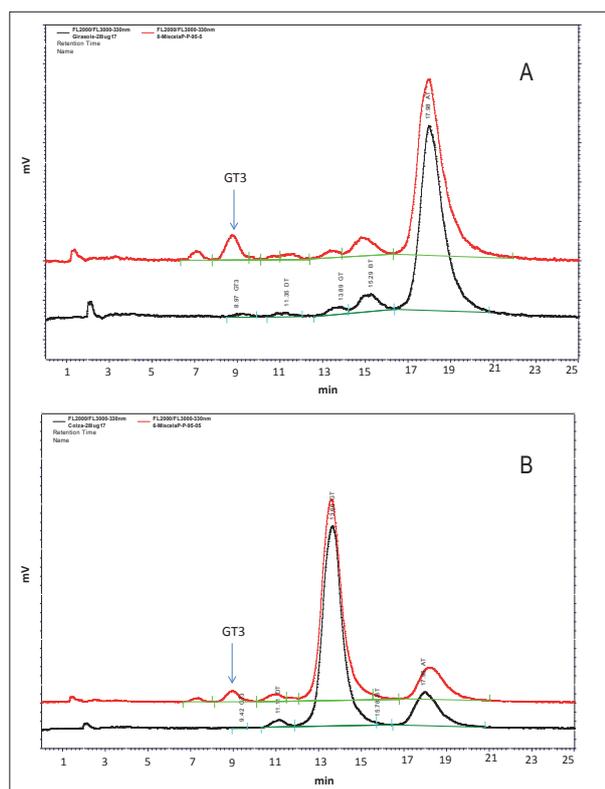
Figure 2 - Chromatograms of sunflower oil (A) and rapeseed oil (B) with the addition of 5% of palm oil (upper line-red) and without addition (lower line-black). GT3 = gammatocotrienol.

Figure 3 - Chromatogram of TAGs in rapeseed oil (A) and sunflower oil (B) with the addition of 2% of palm oil (upper line-black) and without addition (lower line-blue).

MS data were processed using many dedicated software solutions: Xcalibur QualBrowser (Thermo Fisher Scientific, Waltham, MA, USA) software was used to visualise the entire spectra in a raw format; Proteowizard [33] was used to convert raw files to mZmL files that were opened with mMass software (http://www.mmass.org/) and in csv format with R. Data pre-treatment including alignment, data scaling methods (Pareto scaling, auto scaling, range scaling and mean centering) and other treatment (cube or log) and normalization procedures (sum, median and quantile normalization) were done with the free online software Metaboanlyst 4.0 (www.metaboanalyst. ca). Unsupervised and supervised multivariate modelling was performed also using Metaboanlyst 4.0.

A non-targeted approach was used to identify the diagnostic ions responsible for the differences in the mass spectra.

The loadings plot of the supervised partial least square discriminant analysis revealed the abundance of the m/z 850.7841 in PO in comparison to other oils, while the opposite situation was with the m/z 902.8138 in the vegetable oils tested except PO. An attempt to identify these two ions was effectuated using Metlin (metlin.scripps.edu), a repository of metabolite information, as well as tandem mass spectrometry data; it contains over 100000 structures and allows the identification of unknown molecules in pos or neg modes.
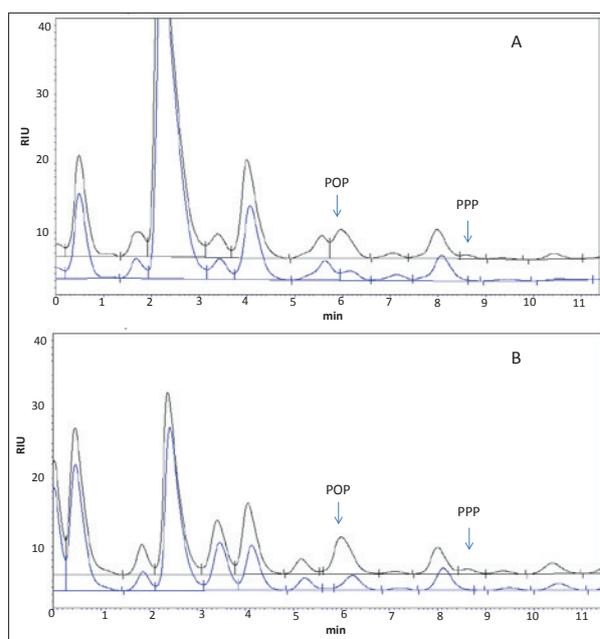
By using Metlin it was possible to annotate the m/z 850.7841 as the ammonia adduct of dipalmitin-olein and the m/z 902.8138 as the ammonia adduct of tri-olein. In the lower interval of the spectra it is possible to note the ions of palmitic-oleic diglyceride (PO, m/z 577.5170), monoglyceride palmitic-glycerol (m/z 313.2728), oleic (m/z 300.2890 [M+NH4]) and oxidized linoleic acid (m/z 297.2517 [M-ox]).

In Figure 4 the full scan spectra of PO (A), sunflower oil (B) and a bakery product containing PO (C) are shown.

The analysis of the 32 food samples (see *2.2 Samples and sample preparation)* with or without PO stated with tocotrienols/TAG's analysis (see 3.4 and 3.5) allowed to define that the ratio between PPO and OOO above 0.2 is correlated to palm oil addition. Other 25 food samples were analysed (see Tab. II): the plot of the PPO and OOO ratio (Fig. 5) of all the samples shows a clear distinction between products with and without PO. All the results obtained with this model fit at 90% with the product labels. Some problems were given from products with cacao or chocolate; the model did not work very well for these items, for which there is the need for in-depth examination; these samples were excluded from the graph in Figure 5.

Anyhow, the detection limit was up to 2% palm oil addition in vegetable oils (sunflower and rapeseed oils tested), so the DART-HR/MS approach can be used as a screening method in order to identify PO food frauds.
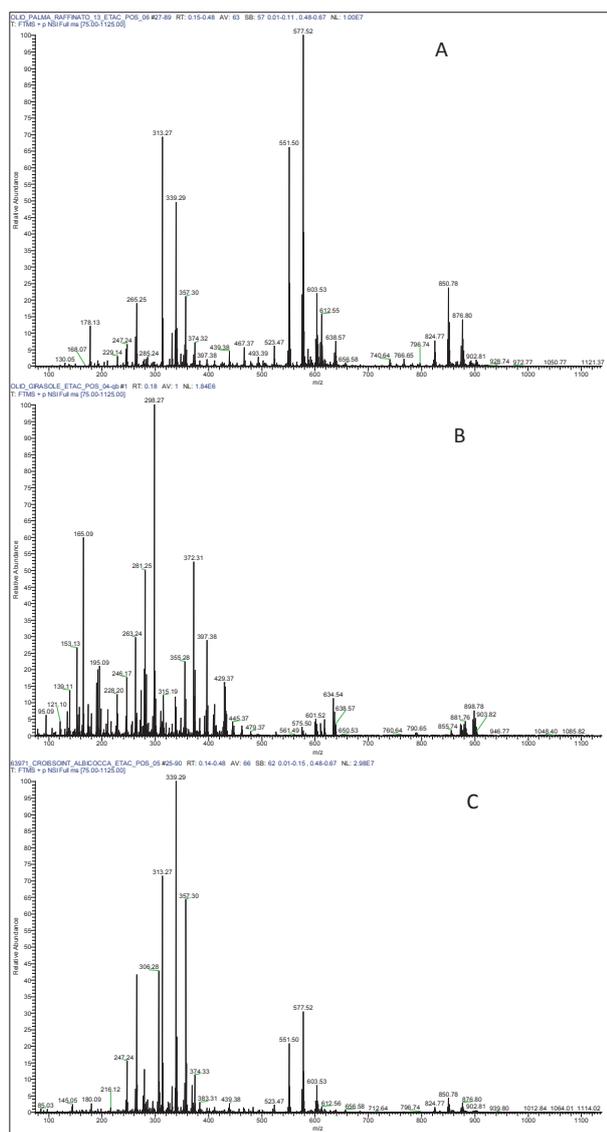
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Figure 4 - Full scan spectra of PO (A), sunflower oil (B) and a bakery product containing PO (C)

3.5 TAG's DART/ION TRAP RESULTS

The pairing of DART with the mass detector is usually performed with a high resolution mass spectrometry utilising the resolution to differentiate the different substances. With a low-resolution mass spectrometry it is possible to enhance the resolving power with the mass-mass technique.

In our work, the DART interface was also coupled with a ion trap, in MS-MS mode. The sample preparation and the DART conditions were the same as illustrated before for DART-HRMS. The sample introduction in DART-MS was achieved placing 5 μL of diluted fat on the tip of a melting point capillary.

As a precursor ion, we chose m/z 824, the ammonium adduct of molecular ion of tripalmitin (PPP+NH4+) that is abundant in palm oil and is scarcely present in other oils: m/z 824 gives, at 40% of collision energy, as product ions m/z 550 and 524 [34]. The presence

Table II - Extra food items analyzed with DART-HRMS

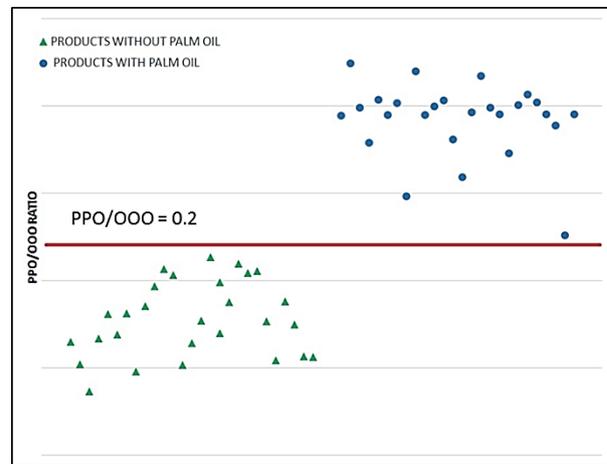| Food item	Number
Biscuit	13
crackers	6
Potatoes snacks	6

Figure 5 - Plot of the PPO and OOO ratio for 54 food samples.

of PO was verified when the ions at m/z 550 and 524 were both equal or above 30% relative intensity in the 300-800 m/z range. In this condition it was possible to detect up to 2% of palm oil addition (sunflower and rapeseed oils tested). Among the 32 real samples analysed, the presence/absence of PO was the same as in the confirmatory methods. In Figure 6 there are the MS-MS spectra of the precursor ion at m/z 824 in the same confectionery product with and without PO in the composition: the product ions at m/z 550 and 524 (highlighted with the arrows) are clearly present in the first formulation only.

DART-ion trap has to be considered a screening method too.

4. CONCLUSIONS

PO has favourable rheological properties; it is one of the cheapest vegetable oils and it is frequently used in food production. After the last consideration on PO for its saturated FA and the presence of 2,3 MCPD, the problem of palm oil detection in food had not been really faced until now. This is only a preliminary work that demonstrates that palm oil detection in food is possible and reliable at a level of more than 2% for screening methods and 1% for confirmatory methods. Using direct analysis in real time (DART) mass detection with both HS mass detector and ion trap, a fast and simple screening is possible; the more time consuming HPLC methods with fluorescence and refractive index detectors for confirmatory analysis are
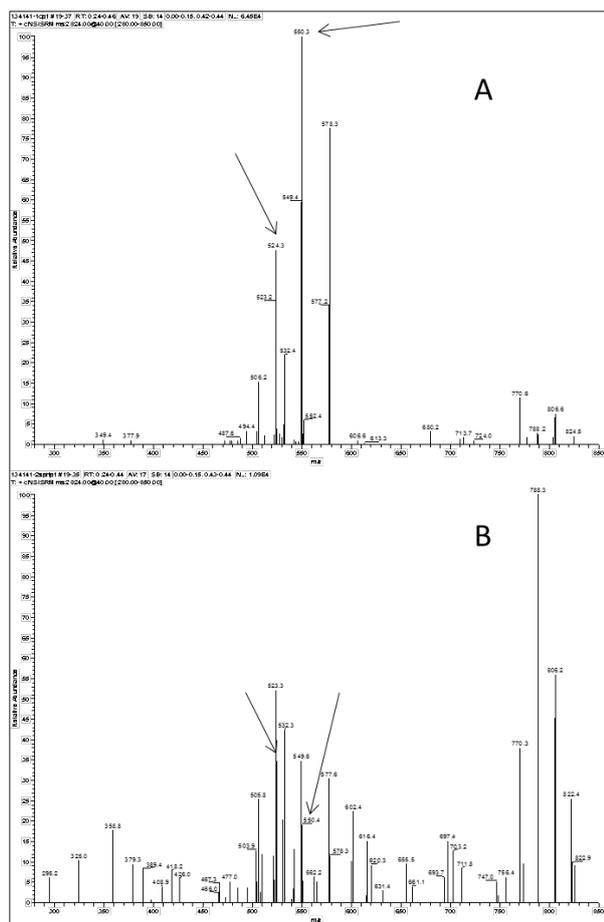
Figure 6 - Ms-Ms spectrum of biscuit with palm oil (A) and without (B): the arrows indicate the monitored product ions

necessary. Further work is necessary to validate both screening and confirmatory methods.

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