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Formation of by-products during chemical interesterification of lipids. Detection and characterization of dialkyl ketones by non-aqueous reversed-phase liquid chromatography-high resolution mass spectrometry and gas chromatography-mass spectrometry

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ABSTRACT

A new class of foreign substances present in the unsaponifiable fraction of vegetable oils undergone to chemical interesterification was systematically investigated. Their chemical structure, corresponding to dialkyl ketones (DAK) molecules, was elucidated both by gas chromatography-mass spectrometry (GC–MS), and liquid chromatography-high resolution mass spectrometry (LC-HRMS). An analytical protocol aimed to qualitative and quantitative detection of DAK molecules in vegetable oils of confectionery industry interest was developed. Being the range of concentration levels to be evaluated dependent on the technological task of interesterification process, the quantitation step was thoroughly examined. All the validation parameters were satisfactory and particularly the concentration determinations were made more reliable by the contemporary use of several quantitation standards. GC–MS and LC-HRMS analytical techniques exhibited comparable performances even if the second one shown better detection sensitivity.

1. Introduction

Interesterification is a common industrial practice aimed to redistribute fatty acids among the triglycerides (TAG) under the influence of a catalyst [1]. Interesterified fats have different physical and functional characteristics and are produced for many purposes like changing overall melting profile, improving compatibility of different triglycerides in solid state, improving plasticity by changing the crystallization behaviour and combining properties of mixed oils and fats [2]. The improved physicochemical characteristics of interesterified blends are described by Zhu et al. [3] and the use of interesterified fats in chocolate and bakery products was recently reviewed [4]. During interesterification process many by-products may be obtained like esters of fatty acids, soaps, free fatty acids, di- and mono-glycerides, tocopherols and esterified sterols [5] that are all species with a chemical structure immediately traceable back to the original fat composition. However, already many years ago foreign peaks in unsaponifiable fraction of chemically interesterified oils were detected and preliminary news of their detection together with a proposal of their structure was presented at the 97th AOCS Meeting in St-Louis in 2006 by R. Verhé of Ghent University [6]. Such extraneous peaks were proposed to be dialkyl ketones (DAK) and the supposed molecular structure was confirmed by the synthesis of some DAK molecules. More recently, a work aimed to distinguish oils interesterified with different methodologies, determined DAKs as neo-formed com-

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ponents useful to discriminate the chemical from the enzymatic interesterification process [7].

As a matter of fact, during the interesterification process a competitive reaction producing dialkyl ketones as by-products can occur. The proposed mechanism of their formation involved a β-keto-ester specie which is an intermediate in the enolate reaction mechanism describing the interesterification process [6]. The intermediate mainly produces the formation of regioisomers of the original TAGs but can partly produce dialkyl ketones as a consequence of a decarboxylation process. The details of this competitive reaction are illustrated in Supplementary Section Fig. 1.

As it can be seen the resulting dialkyl ketone molecules are characterized by a central carbonyl group and by two aliphatic lateral chains coming from the original fatty acids involved in decarboxylation process. Such compounds are present in the unsaponifiable fraction of fats and so, once extracted, may be identified and quantified. The lateral chain length can change according to the vegetable oil undergone to interesterification. In fact, there is a difference between lauric (e.g. coconut oil, palm kernel oil) and non-lauric vegetable oils (e.g. palm oil, shea oil, sunflower oil, cocoa butter) in terms of chain length according to the triglyceride composition.

Their nomenclature [8] is referred to the total carbon atoms present in the two lateral aliphatic chains minus the one lost in decarboxylation process together with the numerical position of the carbonyl group. For example 18-pentatriacontanone ($C_{35}H_{70}O$) indicates a symmetric DAK formed by two C17 aliphatic chains and a central carbonyl group. In alternative, a more synthetic notation is represented by the total carbon atom number C35. Anyway, there may be numerical coincidence of total carbon number coming from combination of differently unsaturated lateral chains. As described by Verhé et al. [6], we use the general formula C*n*-C*m* where *n* and *m* represent the original carbon number of fatty acid chains before decarboxylation. In this way, it is immediately evident the information about the DAK composition; for example the 18-pentatriacontanone with this type of notation corresponds to C18:0-C18:0.

On the basis of the known triglyceride composition of vegetable oils used in confectionery industry it was possible to hypothesize the expected profile of dialkyl ketones that can be potentially formed as by-products during chemical interesterification process:

C8:0-C10:0, C10:0-C12:0, C12:0-C12:0, C12:0-C14:0, C12:0-C16:0, C14:0-C14:0, C14:0-C16:0, typically in a lauric oil like coconut oil and, C16:0-C16:0, C16:0-C18:1, C16:0-C18:0, C18:1-C18:2, C18:1-C18:1, C18:0-C18:1, C18:0-C18:0, typically in non-lauric oils like palm, sunflower, cocoa and shea oil.

Being DAKs neo-formed substances structurally quite distant from known interesterification products it is important to study in detail the conditions of their analytical determination as it is common practice in case of newly formed compounds [9,10]. Lipid transformation was often object of several studies as reported by Berry [11] and Dijkstra [12]. The main goal of our work was the development of both GC–MS and LC-HRMS analytical methods applicable, after a suitable unsaponifiable fraction extraction and purification, to the separation, identification and quantitation of chemically interesterified fats used in confectionery industry. Due to the presence of very few DAK pure standards on the market we tested, at first, the reliability of the identification ability of the GC–MS technique as proposed by the two studies found in literature [6,7]. The second analytical aspect tested was the quantitative determination of DAKs in real samples. This aspect is important in confectionery industry practice but it is complex due to the small number of available pure standards.

The development of an analytical method aimed to DAKs detection in the confectionery industry context is related to raw material quality control. It may be used to check the occurrence of an interesterification process of vegetable oils and in this case is coming along to other methods already present in internal industrial laboratories like the determination of triacylglycerol positional isomers [13] and of *sn*-2 linked fatty acids [14]. In addition it may become the only method able to discriminate between vegetable oils chemically or enzymatically interesterified. The assessment of the type of interesterification applied may be relevant taking into account that sometimes the enzymatic interesterification cannot be used at the same cost of the chemical one and not assure equivalent results.

2. Experimental section

2.1. Materials

Acetonitrile, n-heptane, methylene chloride (HPLC-MS grade), petroleum ether (analytical grade), diethyl ether (analytical grade), ethanol 95% (reagent grade), potassium hydroxide, phenolphthalein indicator (1.0%), anhydrous Na_2SO_4 were purchased from Sigma-Aldrich (St. Louis, MO). Standards of 14-heptacosanone (C14:0-C14:0), 9-octadecanone (C9:0-C10:0), 18-pentatriacontanone (C18:0-C18:0), 11-heneicosanone (C11:0-C11:0) and heptadecylstearate (Wax 35) were purchased from Sigma-Aldrich (St. Louis, MO). Samples of vegetable oils, either original or suitably interesterified, were kindly provided by Soremartec Italia S.r.l (Ferrero Group).

2.2. Vegetable oils chemical interesterification

Vegetable liquid oils normally used in confectionery industry like coconut oil, palm oil, shea oil, and cocoa butter were undergone to batch interesterification process described in literature [5], which is anyway the more recent detailed description of a process widely applied since the forties of the last century [15]. The liquid oil was pumped into batch reactor which was operated under an absolute pressure of <20 mbar. The reactor was a pressure vessel made of steel. The oil was heated to 90 °C and held at this temperature for approximately 60 min to remove the moisture and dissolved air from the oil. The catalyst (sodium methoxide) was added to the oil providing to avoid that air can come into reactor. Its dosage was 0.1% of the oil in the reactor. The reaction was carried out with agitation for 30 min. The reaction mixture was successively cooled and the catalyst deactivated by introducing citric acid. The oil and the aqueous phase was separated in a centrifuge. The reaction product was the bleached under vacuum with activated bleaching clay (0.2%) to remove the residual impurities. The bleached product was filtered and then deodorized. The product after deodorization was cooled to ambient temperature and stored under nitrogen till to successive saponification step and extraction of unsaponifiable fraction. The vegetable oils so interesterified were coconut oil, shea oil, palm oil, cocoa butter, sun flower oil both singularly and mixed together in binary mixtures.

2.3. Unsaponifiable fraction extraction

Extraction procedure was the official procedure made up for the extraction of aliphatic alcohols [16] whose details are reported below.

An amount of 2.5 g of each sample of vegetable oil taken into consideration in this work (coconut oil, palm oil, shea oil and cocoa butter) was weighed and put in a reflux condenser (100.0 mL) together with 50.0 mL of 2.0 N KOH solution in ethanol. A standard solution of DAK C9:0-C10:0 and DAK C14:0-C14:0 (both 100 ppm final concentration) were added to evaluate the recovery efficiency in the case of lauric and non-lauric oil respectively. The solution was heated in a bath oil at 95 °C for 1 h. After cooling the extract was transferred in a separatory funnel (A) and washed with 50.0 mL of ultrapure water. The reflux condenser was washed

with 50.0 mL of petroleum ether which were successively added to the separatory funnel (A). After 1.0 min shaking the water layer was transferred in another funnel (B) and washed three times with 50.0 mL of petroleum ether. All the ethereal fractions were collected together in the funnel (A) and furtherly washed with 50.0 mL of a 1:1 ethanol/water mixture. After checking the neutrality of water/ethanol layer by phenolphthalein indicator, anhydrous Na_2SO_4 was added to optimize dehydration of ethereal layer. The extract was filtered under vacuum and carefully dried. The dry residue was reconstituted with 1.0 mL of methylene chloride in case of direct introduction into the GC or LC system or *n*-heptane for a successive purification step.

2.4. Unsaponifiable fraction purification

The obtained unsaponifiable fraction was submitted to a purification step on a silica SPE column. The conditions were the following: The SPE column (Mega Bond Elut Si, 1.0 g, Agilent Technologies) was previously conditioned with heptane 6.0 mL. Successively 1.0 mL of sample was loaded and eluted by gravity. The bed of the column was dried under vacuum after the elution. 12.0 mL of n-heptane were added as washing step of possibly present *n*-alkanes and discarded. Successively two fractions of 6.0 mL of a fresh prepared mixture heptane:diethyl ether 99:1 were added and eluted by gravity. The final dryness of the column bed was obtained under vacuum. The eluted fractions were collected in a flask, evaporated to dryness and the dry residue was reconstituted with 1.0 mL of methylene chloride.

2.5. GC/MS analysis

GC/MS experiments were performed on a Agilent 6890 N gas chromatograph coupled to Agilent 5937-inert mass spectrometer (Agilent Technologies, Waldbronn, Germany) using a Zebron (ZB-5HT) column, 30.0 m length, 0.25 mm ID, 0.25 μm film thickness (Phenomenex, Torrence, CA). GC conditions were as follows: injector temperature 300 °C, injection volume 1.0 μL in splitless conditions (1.0 min) followed by a split ratio 1:100, flow rate of helium carrier gas 1.2 mL/min. Temperature program: initial temperature 80.0 °C for 1.0 min, ramp to 200.0 °C at 25 °C/min, successive ramp to 290 °C at 10 °C/min and final isotherm of 20 min. Mass detector conditions: ionization mode: EI (70 eV); scan range 50-600 *m/z*; ion source temperature: 230 °C; transfer line temperature: 290 °C.

2.6. LC/HRMS analysis

LC-HRMS experiments were performed on a Ultimate 3000 HPLC (Thermo Scientific, Rodano, Italy) coupled by an Atmospheric Pressure Chemical Ionization (APCI) source to a linear ion trap equipped with a high resolution analyzer (LTQ-Orbitrap, Thermo Scientific, Bremen Germany).

Chromatographic column: RP-C30, 100 mm length, 2.1 mm external diameter, 3.0 μm particle size (Acclaim, Thermo Scientific, Rodano, Italy). A binary mobile phase composed by eluent A (acetonitrile:methylene chloride 95:5) and eluent B (methylene chloride:acetonitrile 95:5) was used. Separation mobile phase conditions: 5.0 min initial isocratic conditions at 100% A, 55.0 min - 50%A + 50%B, 60.0 min - 100%B. Flow: 0.2 mL/min; injection volume: 5.0 μL; column compartment thermostatted at 30 °C.

The LTQ-Orbitrap analyzer with positive ion APCI was used in the mass range 50-700 *m/z*. Vaporizer temperature: 450 °C, sheath gas flow rate: 35 (arbitrary units), aux gas flow rate: 15 (arbitrary units), discharge current: 6 μA, capillary temperature: 250 °C, capillary voltage: 2.0 V, tube lens: 80 V. Resolution: 30,000 (FWHM). Mass accuracy: 5 ppm (without internal calibration). MS/MS collision energy: 30 (arbitrary units).

2.7. Statistical evaluation of final data and validation

In the present work the validation data were obtained following the guidelines of "Laboratory Guides to Method Validation and Related Topics" of Eurachem [17]. Selectivity, trueness, precision, linear range, LOD and LOQ, measurement uncertainty and recovery were evaluated following the statistics described in Miller and Miller [18] and Hubaux and Vos [19]. Calibration curves of recovery and quantitation standards were realized in 6 replicates and each curve was made up by 8 points spanning different concentration intervals depending on the used technique (GC–MS or LC-HRMS).

3. Results and discussion

Few dialkyl ketones pure standards are commercially available. The only way to have all possible DAKs should be their synthesis and purification, but the experimental work necessary to obtain them pure and in substantial amount is prohibitive. So we used the few available ones to develop an extraction and purification procedure and GC and LC chromatographic separation methods taking advantage that all possible DAKs of interest will have structures and physico-chemical properties likely standing between the two available pure standards DAK C9:0-C10:0 and C18:0-C18:0. So we assumed that the available standards were representative of the behavior of all possible DAKs to extraction and chromatographic separation. As a consequence, we chose DAK C9:0-C10:0 (not present in lauric oils) as recovery standard for lauric oils and DAK C14:0-C14:0 (negligibly present in non lauric oils) as recovery standard for non lauric ones. The choose of suitable quantitation standards represented a more complex problem, being the DAK detection sensitivity dependent mainly on lateral chain length and required a careful investigation.

3.1. GC/MS analysis

In Supplementary Fig. 2 the chromatographic separation of the standard mixture (each component 10.0 ppm) is visualized with the relative identifications. As expected there is some difference in intrinsic detection sensitivity depending on the length of lateral aliphatic chains.

The electron ionization (EI) mass spectra of DAK C11:0-C11:0, C14:0-C14:0 and C18:0-C18:0 show a peculiar pattern characterized by two product ions emerging from a *m/z* value distribution typical of aliphatic structures and dependent from DAK lateral chain length. For DAK C11:0-C11:0 the *m/z* values 169, 185, for DAK C14:0-C14:0 the *m/z* values 211, 227 and for DAK C18:0-C18:0 the *m/z* values 267, 283 respectively. In all cases, the numerical difference between each couple of product ions is 16 u independently from the type of DAK involved. In Fig. 1, as an example, the EI mass spectrum of DAK C14:0-C14:0 is reported. The proposed fragmentation mechanism leading, through competitive dissociation reactions, to the structurally significant product ions distant 16 u (denoted by a red star) is illustrated in the boxes in the same figure.

For asymmetric DAKs present in chemically interesterified vegetable oils the fragmentation pathways follow the same scheme underlined for the symmetric ones. For example the DAK C16:0-C18:0, that we found in an interesterified palm oil sample, showed the EI mass spectrum reported in Supplementary Fig. 3A where the couples of product ions (marked with a red star) equally distant of 16 u refer to the lateral chains of different length.

The presence of an unsaturation in the DAK lateral chain introduces a new important fragmentation mechanism in addition to the

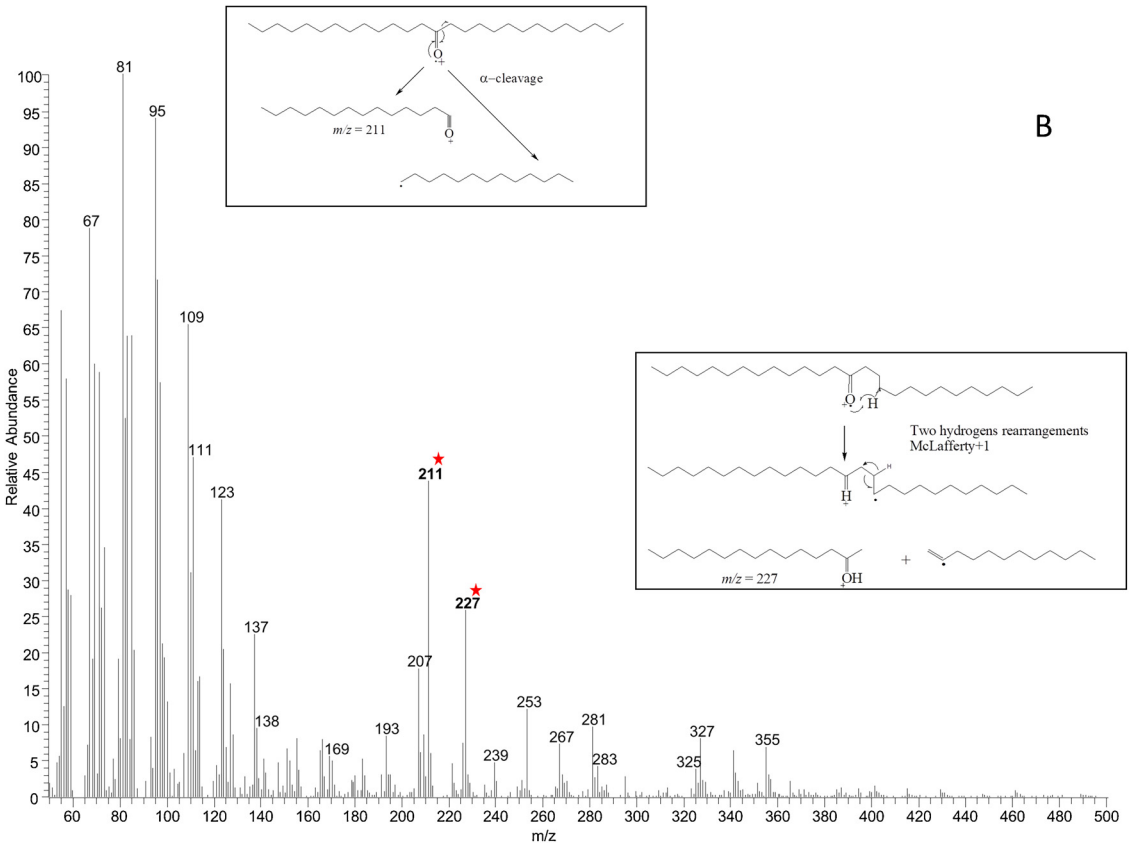

Fig. 1. Electron ionization (EI) mass spectrum of DAK C14:0-C14:0. Product ions involved in the DAK specific fragmentation are marked with red stars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

ones before specified, known as allylic cleavage, involving a radicalization on the double bond followed by a breaking of the bond between the allylic CH_2- group and the rest of the molecule. In Supplementary Fig. 3B the mass spectrum of DAK C16:0-C18:1 (found in a chemically interesterified palm oil sample) is shown.

Based on mass spectrometric data it resulted evident that even in the absence of pure standards the identification of all DAKs in an interesterification process would be easily achievable. As a matter of fact the peculiarity of their EI MS fragmentation spectra allowed to distinguish them unequivocally from any other substance present in the unsaponifiable fraction (sterols, tocopherols, long chain alcohols, etc.).

We applied the method developed for pure standards to real samples and many peaks due to no-DAK molecules were present depending on the type of chemically interesterified vegetable oil. The qualitative recognition of DAKs was always possible due to the specificity of their mass spectra but their quantitation was difficult because of some co-elutions with peaks attributable to predominant components of unsaponifiable fraction were occurring. So a purification step became necessary in order to obtain simpler chromatograms and more reliable quantitation of peaks of interest.

By using SPE silica column and suitable washing and elution steps (see Experimental Section) it was possible to obtain a significant purification of the original unsaponifiable fraction. The high efficiency of purification step is due to the less retention by silica stationary phase of SPE column of DAK molecules in respect to other unsaponifiable components. By modulating the strength of elution solvents it was possible to obtain their selective elution. The Figs. 4 and 5 of Supplementary Section show, as examples, a comparison between chromatograms obtained before and after the purification step of a mixture of sun flower plus shea oil representative of a mixture used in confectionery industry and of a sample of cocoa butter.

3.2. GC–MS data validation

The method selectivity was guaranteed by the specificity of EI mass spectra of DAKs.

In Table 1 linearity parameters, LOD and LOQ values, repeatability and reproducibility percent values corresponding to a low, medium and high concentration level of the calibration curve of each standard are reported.

By increasing concentration, repeatability and reproducibility values, expressed as RSD%, were lowering as expected by homoscedasticity of calibration curve and their percent values were always lower than 20% as fixed by many regulatory agency [20].

As described in the Introduction the quantitative aspects of DAKs determination are important, same validation parameters like trueness, aimed to check the presence of systematic errors, precision, as evaluation of random errors, with their contribution to the measurement uncertainty (accuracy) and recovery were evaluated.

At first the presence of a possible bias (trueness evaluation) due to matrix effect was investigated. As described in Eurachem Method Validation [17], the size of the matrix effect is usually proportional to the signal and changes the slope of the calibration function. So we evaluated the slope ratio of the calibration curves obtained for each available pure standard both added to the unsaponifiable fraction of a not interesterified vegetable oil and dissolved in a solvent. The percent calculated ratios were ranging between 88 and 91% showing a quite low matrix effect. These results made reliable the adoption of calibration curves based on external standards dissolved in a pure solvent allowing to avoid the difficult choice of quantitation standards which otherwise should have to meet two

Table 1
Statistical data of validation of GC–MS results.

Compound	R^2	Slope	Intercept	LOD [ppm]	LOQ [ppm]	Repeatability (RSD%)			Reproducibility (RSD%)		
						5 ppm	15 ppm	50 ppm	5 ppm	15 ppm	50 ppm
C9:0-C10:0	0.9939	$(3.67 \pm 0.13)x10^5$	$(-7.32 \pm 3.12)x10^5$	3.12	5.76	6.50	3.10	2.61	17.7	13.5	4.8
C11:0-C11:0	0.9999	$(7.35 \pm 0.03)x10^5$	$(-6.13 \pm 0.67)x10^5$	1.93	4.43	4.74	4.09	2.12	15.1	10.9	1.2
C14:0-C14:0	0.9973	$(6.18 \pm 0.14)x10^5$	$(-1.36 \pm 0.35)x10^6$	2.50	3.15	12.91	5.15	3.70	12.8	8.88	5.91
C18:0-C18:0	0.9941	$(4.50 \pm 0.15)x10^5$	$(-1.48 \pm 0.38)x10^6$	3.51	4.02	12.56	6.41	2.74	12.7	6.04	6.64

Table 2
Quantitation of added amounts to a not interesterified palm oil sample of pure DAK standards by the mean calibration curves (six replicates) of the same DAKs. For diagonal elements the confidence interval at 95% confidence level are reported.

Concentration of spiked pure standard (ppm)	Quantification standard			
	C9:0-C10:0	C11:0-C11:0	C14:0-C14:0	C18:0-C18:0
	Accuracy of spiked concentration evaluation ($x- \pm t_{(0.05,5)}$ s) (ppm)			
C9:0-C10:0 (12.0 ppm)	**11.9 ± 1.4**	13.7	32.5	73.4
C11:0-C11:0 (11.2 ppm)	9.3	**10.7 ± 3.8**	25.2	60.5
C14:0-C14:0 (14.1 ppm)	5.3	6.0	**14.2 ± 2.3**	34.1
C18:0-C18:0 (11.0 ppm)	2.5	2.7	4.9	**12.4 ± 2.9**

demanding requirements. First they should not be present in real samples and second should have detection sensitivity similar to the various DAKs. The possibility instead to use external standard calibration curves freed us to meet the first requirement whereas the second one could be bypassed using more than one quantitation standards.

In order to check the use of different standard we tested the suitability of the available pure DAKs as quantitation standards. So we spiked them in known quantities to the unsaponifiable fractions extracted from a not interesterified palm oil sample. The aim was to estimate the reliability with which any single standard can quantitate the others (Table 2).

The diagonal elements (bold characters) show that the best evaluation of added amount of each DAK is obviously performed by the same DAK molecule. However, the drastic loss of accuracy shown in the off-diagonal elements confirms the dependence effect of detection sensitivity on the lateral chain length on the measurement of uncertainty and the consequent necessity of using different quantitation standards to reliably quantitate DAKs characterized by lateral chains of varying length.

From these results we drew that in the case of coconut oil we could assume the use of DAK C11:0-C11:0 as acceptable quantitation standard of all DAKs till to C12:0-C14:0 (main components) and DAK C14:0-C14:0 as acceptable quantitation standard of the minor constituent DAKs having longer lateral chains. For palm, cocoa butter and shea oil samples we could assume instead the use the DAK C14:0-C14:0 contemporarily as recovery standard and quantitation standard of the DAKs having lateral chain length till to 16 carbon atoms. For DAKs of longer lateral chains the quantitation standard to be used should be DAK C18:0-C18:0. The accuracy detection obtained by using Wax 35 are not reported because were in all cases highly unsatisfactory.

The recovery of the two chosen surrogates (for lauric and non lauric vegetable oils) were ranging between 78 and 89% and a compensation was operated by using, in the buildup of the calibration curves, the ratio of real chromatographic peak areas to recovery standard area.

3.3. HPLC-HRMS analysis

DAK molecules are highly lipophilic in nature so the best chromatographic mode suitable for their separation was the Non Aqueous-Reversed Phase (NA-RP) combining a lipophilic stationary phase with a mobile phase made up of a mixture of non-aqueous solvents (acetonitrile/methylene chloride in our case) where acetonitrile is the chromatographic weak solvent and methylene chloride is the strong modifier. The stationary phase was a reverse phase C30 instead of the more usual C18, because the global length of DAK molecules is higher than 18 carbon atoms and a reverse phase C30 is more suitable to optimize the retention and separation selectivity.

The predictable elution order depended on the total carbon atom number taking into account that, in the presence of unsaturations, the effective length must be reduced of two carbon atoms for each double bond. In Supplementary Fig. 6, analogously to GC—MS analysis, a chromatographic separation of the standard mixture is reported. The chromatogram is an Extracted Ion Chromatogram (EIC) obtained by extraction of the accurate *m/z* values of the standard molecules (mass tolerance 10 ppm) specified in the box to the right. By comparing LC-HRMS separation of Supplementary Figure6 with that obtained in GC—MS (Supplementary Fig. 2) (both reported in extracted ions) it is possible to note a less pronounced difference of detection sensitivity between the various standard molecules in LC-HRMS. In addition the possibility to extract in high resolution the *m/z* value of each DAK allowed to obtain a visualization of separation free from any interference from noise. So, it was possible to expect in case of real samples separations free from interfering peaks coming from the matrix.

This implied there would be no necessity to purify the unsaponifiable fraction extract.

In fact, the chromatographic separations of DAK constituents of chemically interesterified coconut and palm oil visualized in the same conditions did not show presence of any interfering peaks as reported in Fig. 2 and Supplementary Fig. 7.

Due to the higher detection sensitivity LC-HRMS method was able to detect more DAKs in respect to GC–MS technique.

In addition to HRMS detection mode of DAK molecules just described it was possible to further confirm their identification in MS/MS conditions by exploiting some particularity of their fragmentation behaviour. As it is known the MS/MS spectra achievable in LC–MS are less informative than those obtained by electron ionization but for DAKs it was possible to find some specificity reliably traceable back to their molecular structure.

In fact, DAK molecules undergone to collisions in MS/MS experiment exhibited two typical fragmentation pathways in dependence on the presence of unsaturations in the lateral aliphatic chains. In Fig. 3 the MS/MS spectrum of the DAK C14:0-C14:0 pure standard is reported.

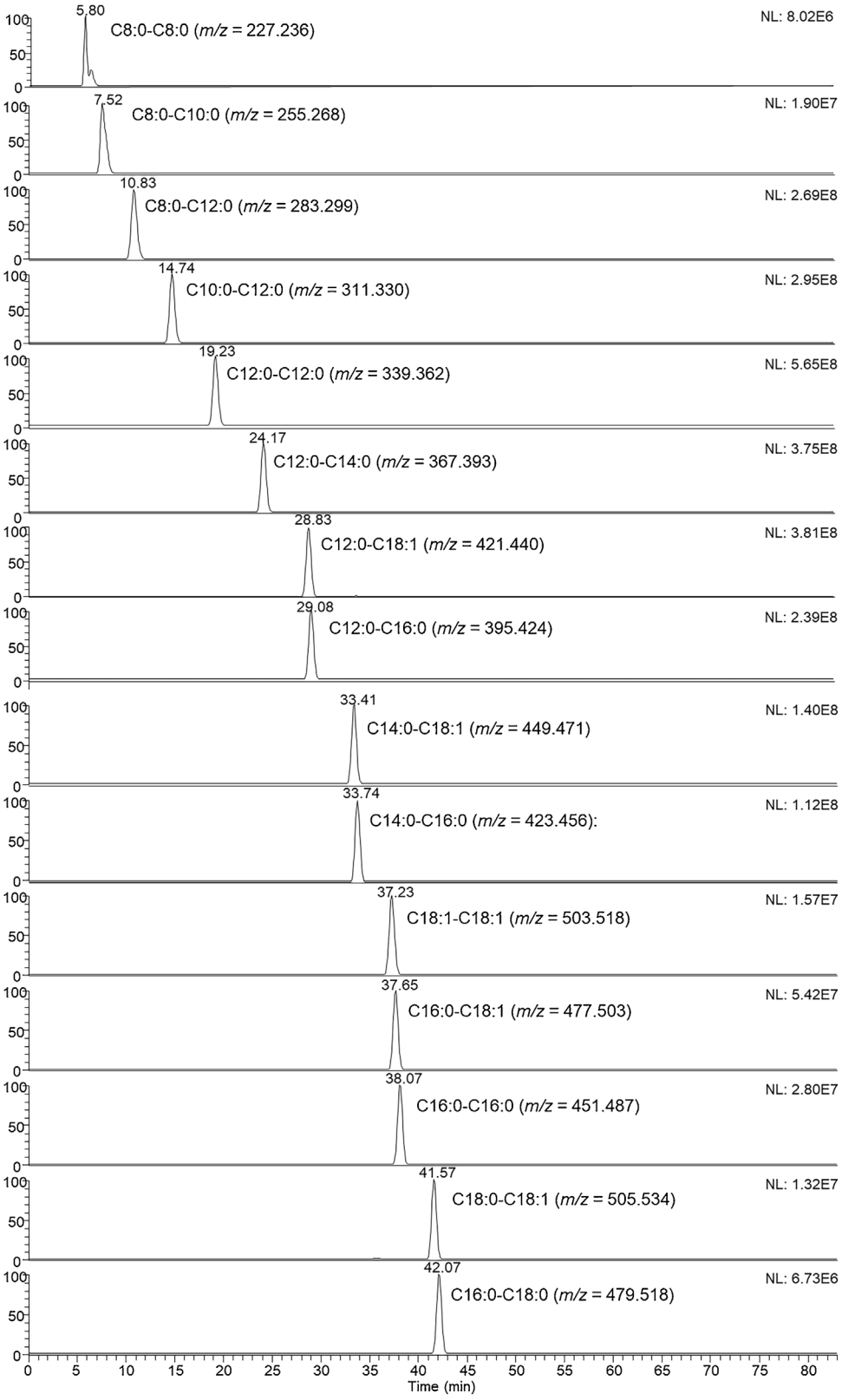

Fig. 2. Extracted Ion Chromatogram of DAK constituents of chemically interesterified coconut oil.

The formation of positive ions in the ionization source occurs through the acceptance of a proton by the central carbonyl group leading to the formation of the $[M+H]^+$ ion at $m/z = 395.424$. In Fig. 3 the MS/MS spectrum of this precursor ion shows as main fragmentation an absolutely uncommon loss of 20.025 u. As it is known, the loss of a neutral fragment needs to meet the requirement that the positive charge remaining on the charged fragment must occupy a position energetically favourable. The loss of 20.025 u does not correspond to any neutral molecule except for HF (but not in high resolution), which is not compatible with DAK structure. So a frag-

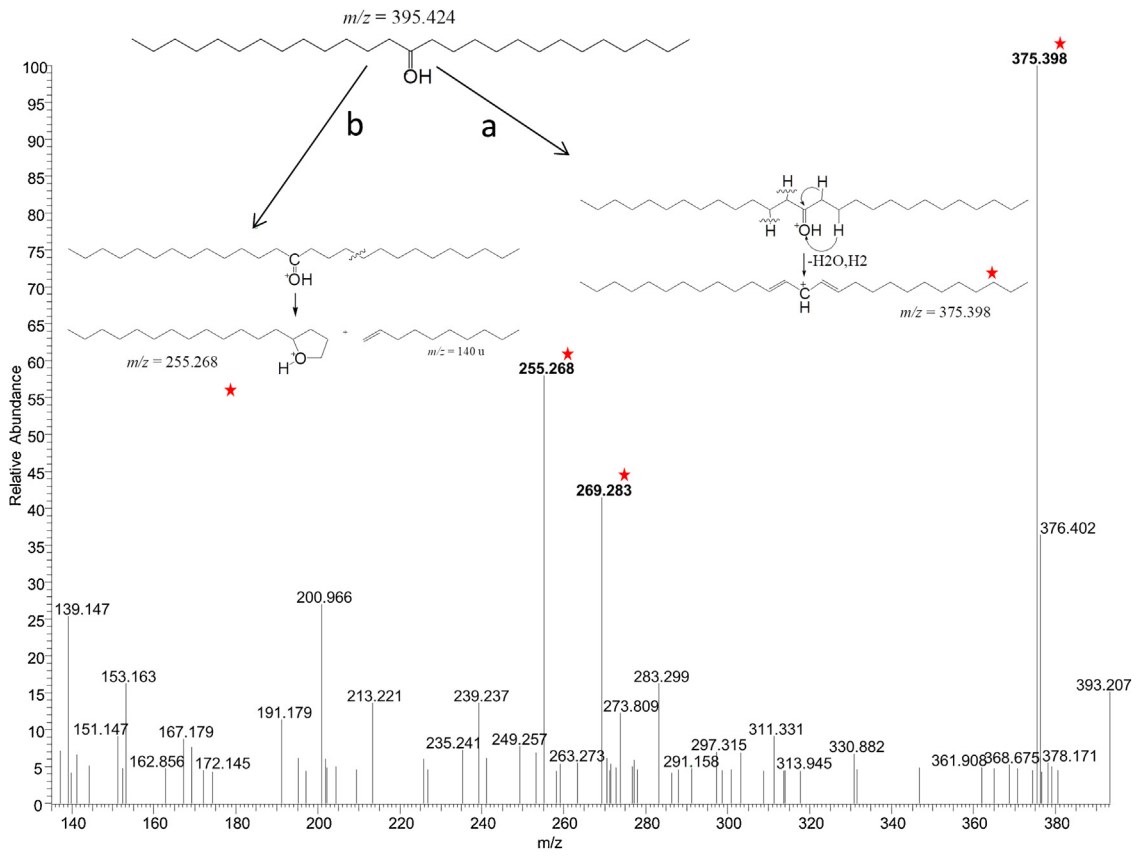

Fig. 3. LC–MS/MS spectrum of the DAK C14:0-C14:0 and proposed mechanism of fragmentation.

mentation pathway which may rationalize this experimental result is a contemporary loss of H_2O and H_2 molecules allowing the formation of an allylic carbon atom able to accommodate the positive charge and stabilize it by resonance (pathway A in Fig. 3).

Two other fragmentation pathways are present where a loss of a part of the lateral chain occurs allowing the formation of a cycle (of five or six atoms) involving the oxygen of the carbonyl group and leaving unchanged the original proton position as illustrated in the pathway B of Fig. 3 for the case of five atoms cycle.

In Supplementary Section Fig. 8 the MS/MS spectrum of DAK C18:0-C18:0 ($[M+H]^+$ ion at $m/z = 507.549$) is reported to highlight the analogy of behaviour with DAK C14:0-C14:0. There is the same neutral loss of 20.025 u and the product ions at $m/z = 311.331$ and 325.347 represent respectively the loss of pieces of lateral chain leading to the formation of the cycles of 5 and 6 atoms with the oxygen of carbonyl group.

Several DAKs identified in chemically interesterified vegetable oils have lateral chains with one or two unsaturations. For these DAKs the MS/MS spectra are different and present as overwhelming fragmentation the loss of H_2O molecule as exemplified in Supplementary Fig. 9 where the MS/MS spectrum of the DAK at $m/z = 505.534$ (DAK C18:0-C18:1) is reported.

The proposed fragmentation scheme rationalizing this experimental evidence makes reference to the high mobility of hydrogen atoms due to the vibrational modes activated by the absorbed collision energy. Their mobility can allow the change of position of the double bond and a contemporary start of a keto-enol tautomery involving the protonated carbonyl group (box of Supplementary Fig. 9). In this way after the H_2O molecule loss the positive charge is located in allylic position and stabilized by resonance with two conjugated double bonds.

Operating in Single Reaction Monitoring (SRM) mode by setting up the suitable collision energy for the precursor ion $(M+H)^+$ it is possible to draw further structural information by recording the formation of the product ion $[(M+H-H_2O)]^+$ for the unsaturated DAKs, the product ions $[(M+H-H_2O+H_2)]^+$ and $[(M+H-C_nH_{2n})]^+$ for the saturated ones. However in these conditions the detection sensitivity was lower than that obtained in high resolution detection of precursor ions so this last detection mode is recommended for the determination of low DAK contents.

3.4. LC-HRMS data validation

The selectivity was high due to high resolution mass spectrometry detection.

As already reported for GC–MS technique in Table 3 linearity parameters, LOD and LOQ values, repeatability and reproducibility percent values corresponding to a low, medium and high concentration level of the calibration curve of each standard are reported.

LOQ values and reproducibility percent values are better than those obtained in GC–MS confirming that the technique LC-HRMS is better for real samples having low DAKs concentration.

As regards the quantitative aspects the same considerations described in the GC–MS section were also here valid and were leading to analogous results (not reported).

Finally we applied the analytical protocols both GC–MS and LC-HRMS, before described in all their details, to the analysis of some real samples and we choose coconut, palm and shea oil of different degrees of interesterification. The aim was to compare the quantitative performance of the two techniques and their congruence. Recovery and quantitation standards for lauric and not lauric vegetable oils were already previously defined.

Table 3
Statistical data of validation of LC-HRMS results.

Compound	R^2	Slope	Intercept	LOD [ppm]	LOQ [ppm]	Repeatability (RSD%)			Reproducibility (RSD%)		
						0.25 ppm	3 ppm	10 ppm	0.25 ppm	3 ppm	10 ppm
C9:0-C10:0	0.9989	$(6.76 \pm 0.05)x10^7$	$(-5.36 \pm 0.70)x10^6$	0.11	0.18	8.23	4.30	1.16	6.32	4.89	2.14
C11:0-C11:0	0.9975	$(7.18 \pm 0.07)x10^7$	$(7.43 \pm 2.22)x10^6$	0.14	0.22	4.38	1.76	0.84	7.73	5.66	4.36
C14:0-C14:0	0.9981	$(4.36 \pm 0.06)x10^7$	$(-5.74 \pm 1.51)x10^6$	0.17	0.26	7.20	3.70	0.78	8.16	5.07	7.45
C18:0-C18:0	0.9968	$(2.59 \pm 0.02)x10^7$	$(-3.00 \pm 0.43)x10^6$	0.17	0.30	3.03	2.23	1.44	11.46	3.97	1.87

Table 4
GC vs LC comparison of total DAK content in chemically interesterified samples.

	Total DAK content (g/kg) ± SD	
	GC	LC
Coconut oil sample	2.9 ± 0.15	2.7 ± 0.39
Shea oil sample	0.75 ± 0.04	0.82 ± 0.06
Palm oil sample	0.080 ± 0.003	0.082 ± 0.005

The quantitation data reported in Table 4 are the mean of three replicates corresponding to three parallel extractions of each vegetable oil.

4. Conclusions

Dialkyl ketones are an important class of compounds newly individuated in the unsaponifiable fraction of vegetable oils formed during chemical interesterification. The work of Vehrè et al. [6] was largely preliminary and the more in depth study of Mariani and Bellan [7] was oriented essentially to distinguish chemical from enzymatic interesterification process of vegetable oils. No other works appeared in literature about this argument. The present work is a systematic analytical study of DAKs present in chemically interesterified vegetable oils either by GC–MS or by LC-HRMS. A detailed study of their EI mass spectra have shown the central role of GC–MS technique in identifying their chemical structure. Anyway extracted unsaponifiable fraction requires a purification step to be analysed by this technique. Alternatively LC-HRMS, taking advantage on high resolution detection, has shown better sensitivity and no need of purification step. LC-tandem MS technique showed less sensitivity but could be useful to confirm the DAKs identification.

The validation data, worked out beginning from the available standards, was satisfactory for both techniques.

The two analytical protocols showed complementary characteristics and may be used either alone or combined for the determination and quantification of DAKs in the unsaponifiable fraction of all chemically interesterified vegetable oil typically used in confectionery industry. Once retention times and identity of all the possible DAKs are known and after careful purification of the unsaponifiable fraction the analysis may be assessed by GC-FID technique.

Conflict of interest

Authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2018.11.001.

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