Development and validation of a rapid method to quantify neutral lipids by NP-HPLC-charged aerosol detector

M.R. Infantes-Garcia^{*}, S.H.E. Verkempinck, J.M. Guevara-Zambrano, M.E. Hendrickx, T. Grauwet^{**}

Laboratory of Food Technology, and Leuven Food Science and Nutrition Research Centre (LFoRCe),
Department of Microbial and Molecular Systems (M²S), KU Leuven, Kasteelpark Arenberg 22, PB 2457,
3001 Leuven, Belgium.

Author's email addresses:

M.R. Infantes-Garcia: marcos.infantes@kuleuven.be

S.H.E. Verkempinck: sarah.verkempinck@kuleuven.be

J.M. Guevara-Zambrano: jessica.guevara@kuleuven.be

M.E. Hendrickx: marceg.hendrickx@kuleuven.be

T. Grauwet: tara.grauwet@kuleuven.be

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^{*}author whom correspondence should be addressed during submission process:

21 marcos.infantes@kuleuven.be

22 +32 16 37 65 53

23 ****author whom correspondence should be addressed post-publication:**

24 tara.grauwet@kuleuven.be

25 +32 16 32 19 47

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Abstract

The study of triglycerides gastrointestinal fate is of main importance due to their nutritional and health implications. In this matter, there is a need for a specific, reliable and fast method to quantify lipid digestion products. The HPLC-CAD method developed in this work proposes a fast manner to simultaneously quantify triolein, *sn*-1,2/2,3-diolein, *sn*-1,3-diolein, *sn*-2-monoolein, *sn*-1/3-monoolein and oleic acid. First, the HPLC separation was optimized by exploiting the elution gradient program and for quantification, optimal CAD detector settings were selected: evaporator temperature of 35 °C and power function value equal to 1.6. Second, the optimized chromatographic method was validated for all analytes. Limits of detection and quantification ranged between 0.8 and 7.3 ng, and between 2.7 and 24.4 ng, respectively. A wide linear range was determined for the six analytes and their calibration curves presented an excellent linear fit ($R^2 = 0.999$, y-intercept = 0.5 – 2.6%, and $RSD \leq 8.4\%$). Accuracy varied between 93 and 114%, while precision values ranged between 0.4 and 2.3%. Finally, the developed HPLC-CAD method was successfully applied to study the digestive fate of an emulsion during *in vitro* digestion. The proposed HPLC-CAD method constitutes an interesting analytical platform to gain insight into the molecular mechanism of lipid digestion.

Keywords

Triolein; oil; HPLC; charged aerosol detector; CAD; method validation; *in vitro* lipid digestion

1. Introduction

Lipids are constituted by a very complex group of hydrophobic compounds. According to the LIPID MAPS classification, most lipid classes found in foods correspond to triglycerides. Triglycerides are composed of a glycerol moiety and three fatty acyls (Fahy et al., 2011). This lipid class has received special attention in the last decades due to associated nutritional and health implications. A balanced triglyceride intake can provide energy, essential nutrients and/or lipophilic nutritional compounds (Yaqoob, 2013). Nevertheless, their overconsumption is negatively associated with the prevalence of obesity and other metabolic diseases (Miller et al., 2005). Therefore, it is critical to gain insight into lipid digestion kinetics and its mechanism to influence related processes, such as absorption of lipophilic vitamins or induction of prolonged satiety effects. These latter processes can be significantly impacted by the lipolysis rate and extent. In this aspect, *in vitro* digestion models have been widely employed in the last two decades due to their lack of ethical constraints, high throughput and reproducibility as well as potential to interrupt the digestion process at any point of interest (Lucas-González et al., 2018).

Apart from the simulation of digestion conditions, an analytical platform is required to quantify the release of digestion products. Specifically for lipid digestion, the most common quantification technique is the titrimetric determination of free fatty acids (FFAs) released from triglycerides (TAGs) after lipase hydrolysis over time, at pH values close to neutral (McClements and Li, 2010). This method has the advantage of online measurement of FFA release, and requires an automatic titration system only. Nevertheless, any compound present in the media reacting with the titrant may lead to significant deviations (limited specificity). Another drawback of this technique is related to its low sensitivity (Hasan et al., 2009). Other methods quantifying lipid digestion products include the use of chromogenic, fluorogenic, turbidimetric or radiometric assays. These latter methods are either expensive, tedious, or time-consuming. An extensive revision of these methods was compiled by Hasan et al. (2009).

The most sensitive and specific methods to quantify (lipid) digestion products correspond to chromatographic techniques. An important advantage of chromatographic techniques is their capacity to

distinguish between different compounds at a molecular level. This is of major importance since lipid digestion not only involves the generation of FFAs, but also intermediate digestion products such as mono- (MAGs) and diglycerides (DAGs). In some cases, thin layer chromatography (TLC) coupled to gas chromatography (GC) was applied to quantify concentrations of FFAs, MAGs, DAGs and TAGs after extraction (Couëdelo et al., 2015). Although TLC is a simple and efficient method to separate lipid classes, it lacks adequate precision in terms of repeatability. In other cases, researchers used high performance liquid chromatography (HPLC) coupled to different detectors. For the quantification, diode array ultraviolet (UV) and refractive index (RI) are typical detectors found for HPLC analysis. The former one, however, would require a high load of analyte because neutral lipids do not efficiently absorb UV radiation (Haidar Ahmad et al., 2019). RI detectors have limitations in terms of gradient elution programs utilization. Therefore, studies regarding HPLC quantification of lipolysis products preferably used aerosol detectors (e.g. evaporative light scattering detector, ELSD). These have been claimed to be mass-sensitive detectors since they provide a universal response (Hutchinson et al., 2011). ELSD was previously utilized to quantify FFAs, MAGs, DAGs, and TAGs during *in vitro* digestion experiments (Carrière et al., 1997; Verkempinck et al., 2018). An interesting alternative to ELSD is a charged aerosol detector (CAD). CAD has a superior performance compared to ELSD with respect to sensitivity, linear dynamic range, and reproducibility (Gamache, 2017). In this aspect, we found one study in which the authors evaluated HPLC elution programs commonly used for the quantification of lipid classes and lipid molecular species coupled to a CAD (Moreau, 2006). However, the detection of lipid isomers derived from the hydrolysis of triglycerides was not explored in the latter study.

In the present work, we aim to develop, validate and apply a HPLC-CAD method as a quantitative tool for triolein digestion and its hydrolysis products, including diolein and monoolein regioisomers. We selected triolein and its hydrolysis products since triolein is the most important triglyceride present in many vegetable oils such as olive, canola or high oleic sunflower oil (Karupaiah and Sundram, 2007). In addition, triolein is a pro-chiral molecule meaning that isomers can be formed when cleaved. Quantification of

diverse intermediate and final lipid digestion products could facilitate gaining insight into the kinetics and molecular mechanism of lipolysis. This insight **would be even more challenging** if the substrate (oil) (e.g. sunflower, soybean, or olive oil) is composed of a complex mixture of triglycerides (e.g. 1,2-dilinolein-3-olein, 1-linolein-2,3-diolein, 1,2-diolein-3-palmitin, or 1,2-dilinolein-3-palmitin) since this would result in **a higher number** of lipolysis products (Karupaiah and Sundram, 2007). Besides challenges at the level of compound separation, the quantification of all these possibly formed analytes would be complicated. In addition, the validation of this method for analytes produced from the hydrolysis of a complex oil would be rather difficult due to the high number of lipid standards needed to perform the tests. Some of these lipid standards are even not commercially available. Therefore, we conclude that the method developed and validated in this work is suitable for fundamental studies in which single-triglyceride oils are employed.

2. Materials and methods

2.1 Materials

Lipid standards were acquired from Larodan (Solna, Sweden). Triolein (> 99 %) was purchased from Acros Organics (Geel, Belgium) and stored at -20 °C with a nitrogen headspace. Sodium taurodeoxycholate (NaTDC) (> 95 %) was obtained from Cayman Chemical Company (MI, USA). Rabbit gastric extract was bought from Lipolytech (Marseille, France) and a sample of pancreatic extract was kindly donated by Nordmark (Uetersen, Germany). The remaining reagents were purchased from Sigma-Aldrich (Diegem, Belgium), except for NaHCO₃, NaCl, H₂SO₄, KH₂PO₄, ethanol and trimethylamine (Fisher Scientific, Merelbeke, Belgium); KCl, MgCl₂(H₂O)₆, acetone, heptane, ethyl acetate and trichloroacetic acid (Acros Organics, Geel, Belgium); HCl, diethylether and iso-propanol (VWR, Leuven, Belgium).

2.2 Instrumentation

The separation of neutral lipids was performed using a HPLC system composed of a quaternary pump, online degasser, automatic sampling device and column compartment (Agilent Technologies 1200 Series, Diegem, Belgium). The HPLC system was fitted with a monolithic silica column (Chromolith Performance

Si, 100-4.6 mm, Merck, Darmstadt, Germany) including a guard column (Merck, Darmstadt, Germany). After each run, a needle wash step with diethylether : heptane (1:1) was performed. The column temperature was set at 40 °C using an external oven to avoid crystallization of lipids inside the column (Chromaster 5310, VWR, Hitachi Ltd., Tokyo, Japan). Analytes were detected using a charged aerosol detector (CAD; Corona Veo, Thermo Fisher Scientific, Geel, Belgium). The evaporator temperature of the CAD can be set at either 35 °C or 50 °C, so both temperature settings were evaluated in this work. The gas pressure was set at 5.5 bar as recommended by the supplier.

2.3 HPLC-CAD method development

The HPLC-CAD method was implemented to obtain an adequate resolution of all analytes, stable baseline signal, and wide dynamic linear range. Lipid standard solutions of triolein; *sn*-1,2/2,3-diolein; *sn*-1,3-diolein; *sn*-2-monoolein; *sn*-1/3-monoolein and oleic acid were prepared in diethylether:heptane (1:1) to perform the following experiments.

2.3.1 HPLC gradient program optimization

Elution of the different neutral lipids was performed using a quaternary gradient consisting of isooctane (solvent A), acetone:ethyl acetate (2:1 v/v) containing 0.02 % (v/v) of acetic acid (solvent B), propan-2-ol:water (85:15 v/v) containing 0.05 % (v/v) of both acetic acid and triethylamine (solvent C) and propan-2-ol (solvent D). The initial flow rate was set at 1 mL min⁻¹ and was increased to 1.5 mL min⁻¹ during the cleaning step of the gradient program. The first program employed in this study was the one proposed by Graeve & Janssen (2009). From this gradient, the proportion between solvents and elution times were modified to reduce the program duration, improve the analytes resolution and stabilize the baseline signal (Fig. 1). For this purpose, 5 µL of a mixture containing 0.1 mg mL⁻¹ of each lipid standard and blank samples (diethylether:heptane (1:1)) were injected in the system.

2.3.2 CAD evaporator temperature selection and power function optimization

The detection of all analytes by the CAD consists of three main steps. First, dry analyte particles are formed by nebulizing and evaporating the eluent coming from the HPLC. Second, the analyte particles are collided with a stream of charged N₂ to transfer the charge to these particles. Third, the charge of the analyte particles is measured in a sensitive electrometer which produces a signal directly proportional to the mass of the analyte present in the system (Almeling et al., 2012; Schilling and Holzgrabe, 2020). The CAD equipment available at our research unit allows detection at two evaporator temperatures (35 or 50 °C). These conditions were compared to select the one resulting in a higher overall signal magnitude. This test was done by injecting 5 µL of a mixture containing 0.1 mg mL⁻¹ of each lipid standard.

The raw CAD signal is based on the electrical current measured in the analyte particles. The raw signal is then mathematically transformed into a new signal which is the one reported in the chromatogram. The relation between the raw signal and sample concentration is proportional, yet not linear as the signal depends on the surface of analyte particles and not on the volume (Haidar Ahmad et al., 2019). To increase the apparent linear range of the calibration curves, the detection signal can be optimized by employing a power function transformation as shown in the following equation:

$$S_1 = S_0^{PFV} + k \quad (1)$$

In equation (1), S_1 represents the transformed signal, S_0 the raw signal, PFV the power function value, and k is a constant. To find the optimal PFV, individual lipid standards were injected onto the system aiming to range the amount on column (AOC) of all analytes between 25 and 2500 ng (6 AOC values). Afterwards, we calculated a response factor which relates the AOC and peak area (peak area/AOC). We selected the PFV associated with the wider dynamic linear range that can be obtained for all analytes simultaneously.

2.4 HPLC-CAD method validation

The limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, and quantitation range of the six analytes were evaluated as validation parameters of the established chromatographic

method. Acceptance criteria recommended by FDA validation guidelines were considered to evaluate the method performance (U.S. Food and Drug Administration, 2018). All details about this validation process are listed in Table 1. Experiments for each validation parameter were conducted in triplicate.

2.5 HPLC-CAD method application

The improved and validated chromatographic method was tested in the context of an *in vitro* digestion experiment. One emulsion was prepared and digested under *in vitro* conditions to evaluate the recovery of triolein and its hydrolysis products as a function of digestion time.

2.5.1 Emulsion formation

Emulsions were formulated with triolein (5 % w/w), the surfactant sodium taurodeoxycholate (1 % w/w) and Milli-Q water (94 % w/w). First, emulsion ingredients were blended in a high-shear mixer (Ultra-Turrax T25, IKA, Staufen, Germany) at 13500 rpm for 5 min to form a pre-emulsion. Second, this mixture was subjected to one cycle of homogenization at 100 MPa in a high-pressure homogenizer (STANSTED SPCH-10, Homogenising Systems, Harlow, UK) to form a fine emulsion. This fine emulsion had a volume-weighted particle size $d(4,3)$ of $0.90 \pm 0.04 \mu\text{m}$.

2.5.2 Static *in vitro* digestion

We selected eight independent digestion moments during the small intestinal phase (5; 10; 15; 30; 45; 60; 90; 120 min after addition of pancreatic enzymes) to evaluate the production of lipolysis species. For this purpose, the standardized protocol of the international network INFOGEST was utilized (Brodkorb *et al.*, 2019). A gastric and small intestinal phase were considered since these are the main compartments where lipid digestion of liquid foods take place.

Gastric phase: In a dark vial, 125 μL of emulsion was mixed with 125 μL of Milli-Q water (to mimic saliva dilution in oral compartment), 200 μL of simulated gastric fluid set at pH 3, and 5 μL of a 15 mM CaCl_2 solution. Subsequently, 15 μL of a rabbit gastric extract (RGE) solution containing rabbit gastric lipase was added to simulate gastric lipid digestion. RGE was dissolved in Milli-Q water to reach a gastric lipase

activity of 60 U mL⁻¹ in the gastric phase (tributyrin-based). After that, HCl (50 mM) was added to reach a pH value of 3. Finally, enough Milli-Q water was added to reach a final chyme volume of 500 µL. The headspace of each vial was filled with nitrogen. Afterwards, the vials were incubated at 37 °C and gastric mechanical agitation was simulated with an end-over-end rotator (40 rpm).

Small intestinal phase: Vials from the gastric phase were opened and a series of solutions were added to the 500 µL of chyme. Exactly 200 µL of simulated intestinal fluid set at pH 7, and 40 µL of a 15 mM CaCl₂ solution were added, followed by 75 µL of a bile salt solution (10 mM in the chyle) and 125 µL of pancreatin (2000 U mL⁻¹ of chyle, lipase activity based on tributyrin). Afterwards, NaOH (50 mM) was included in the reaction mixture to reach a pH value of 7. The total volume was made up to 1 mL with Milli-Q water. The headspace of each vial was once more filled with nitrogen. Hereafter the vials were incubated at 37 °C while being agitated with an end-over-end rotator (40 rpm). Lipid digestion in the small intestinal phase was inhibited by adding 10 µL of 100 mM Orlistat and 10 µL of an 4-bromophenylboronic acid solution (1 M in methanol) to inhibit gastric lipase and pancreatic lipase, respectively. All samples were kept on ice prior to being stored in a freezer at -40°C.

2.5.3 Quantification of lipid digestion products

From triolein (TAG) hydrolysis, diverse hydrolysis products were detected and quantified: *sn*-1,2/2,3-diolein (*sn*-1,2/2,3-DAG); *sn*-1,3-diolein (*sn*-1,3-DAG); *sn*-2-monoolein (*sn*-2-MAG); *sn*-1/3-monoolein (*sn*-1/3-MAG) and oleic acid (FFA). All these neutral lipids were first extracted from the digested samples according to the method described by Infantes-Garcia et al. (2020), and then quantified by means of the HPLC-CAD implemented and validated as described in Sections 2.3 and 2.4.

3. Results and discussion

3.1 HPLC-CAD method development

The chromatographic method proposed in this work has been optimized starting from method (elution program and HPLC column) proposed by Graeve & Janssen (2009). Therefore, we first focused on

optimizing the analytes resolution through adaptation of the gradient program (Section 3.1.1). Afterwards, optimization of detector settings was performed to maximize and linearize the obtained signals (Section 3.1.2).

3.1.1 Gradient program optimization

Analytes were separated through the application of a monolithic silica column as recommended by Graeve and Janssen (2009). The solid phase in monolithic columns consists of silica rods instead of spherical particles typically found in conventional columns. This difference in solid phase structure creates a higher porosity allowing a lower back-pressure compared to conventional columns (Graeve and Janssen, 2009). The lower back-pressure permits higher solvent flow rates and so that analytes can elute faster and, thereby resulting in shorter gradient elution programs.

The first gradient program tested was the one proposed by Graeve & Janssen (2009) (Fig. 1a). They proposed a HPLC-ELSD method to separate and quantify lipids of different polarity. This gradient program starts with a non-polar solvent (i.e. isooctane) and is followed by an increased mobile phase polarity over the time. In solvent B and C, additives acetic acid and triethylamine are added, respectively. These additives reduce interactions between the stationary phase and relatively polar analytes (e.g. fatty acids or monoolein), promoting an efficient elution of these analytes (Li et al., 2010). Graeve & Janssen (2009) targeted to apply the method in marine zooplankton samples, which contain a wide diversity of lipid classes. In our case, we aimed to separate neutral lipids (one lipid class) originating from the hydrolysis of triolein, including intermediate products isomers. Fig. 2a was obtained by injecting a standard mixture composed of six analytes and shows that the method of Graeve & Janssen (2009) separates neutral lipids by species type (tri-, di-, mono-glyceride, and fatty acid). Consequently, regioisomers of diolein and monoolein co-elute, and so cannot be individually quantified. After doing a series of modifications to the program suggested by Graeve & Janssen (Fig. 1b-f), an excellent separation of diolein regioisomers and relatively good separation of monoolein regioisomers was obtained (Fig. 2b). This final gradient program was achieved by changing the proportion of solvents (mainly solvents B and C) and by omitting irrelevant

cleaning steps (Table 2). The other elution programs tested, are also presented in a table format in Supplementary material (Tables S1-S6). In the end, the elution program time was reduced from 35 to 20 min (Fig. 2a and b). In addition to separation of analytes, signal baseline stabilization was also desired. Fig. 2c shows chromatograms of blank injections using different elution programs. It can be observed that the final program (Fig. 2c V) presents a more stable baseline compared to the other programs tested (Fig. 2c I-IV).

3.1.2 CAD evaporator temperature selection and power function optimization

The evaporator temperature determines not only the efficiency of the mobile phase removal from the nebulized analyte particles, but also the sensitivity of the detector for semi-volatile compounds (Schilling and Holzgrabe, 2020). We selected the optimal evaporator temperature based on the maximal detector signal. This was done by comparing individual and total peak areas detected for the six analytes. As depicted in Fig. 3, oleic acid was the analyte most depending on the evaporation temperature. This was anticipated given that for semi-volatile compounds such as oleic acid and monoolein, with a boiling point of 286 and 240 °C, respectively (U.S. Environmental Protection Agency, 2021), volatility determines particle formation. Therefore, a higher evaporation temperature leads to a decreased signal intensity (Schilling et al., 2018). For non-volatile compounds such as triolein and diolein having a boiling point above 500 °C, the signal intensity depends on the particle size distribution of the dried aerosol. This particle size distribution can vary depending on the particle density, an effect that is negligible in nature. Moreover, the charging process occurring inside the CAD is independent of analyte properties. For this reason, we observed a very similar response for triolein and diolein irrespective of the evaporation temperature used (Fig. 3) (Górecki et al., 2006).

After defining the optimal evaporator temperature (i.e. 35 °C), of the two available temperatures (35 °C or 50 °C), the next step was to improve the linearity of the analyte responses. In general, all aerosol detectors generate calibration curves with non-linear behaviors. Thus, it is necessary to check the curve fit and pay special attention to low analyte concentrations (Schilling et al., 2018). This means that the traditional

evaluation of the determination coefficient ($R^2 \approx 1$) does not necessarily imply a good linear fit as this regression technique gives more weight to the high concentrations within the calibration curve. For this reason, the response factor (peak area/AOC) was calculated and plotted versus the AOC (ng) using different power function values (PFV) per analyte (Fig. 4). PFV levels ranging from 1 to 1.8 were evaluated. More information about the meaning and use of PFV can be found in Section 2.3.2. We anticipated that by varying the PFV level, the linear range of the analytes could be increased.

Fig. 4 shows the effect of the PFV on the linearity of signals from triolein and its hydrolysis products in a qualitative manner. However, a quantitative approach is needed to decide which PFV level can be used for the simultaneous quantification of the six analytes. Therefore, the relative standard deviation (RSD) was calculated after subjecting each set of points shown in Fig. 4 to linear regression. The output of this analysis is illustrated in Fig. 5. The larger the deviation from linear behavior, the larger the % of RSD magnitude. From these results, we selected a PFV of 1.6 since this value presented the lowest RSD for the majority of responses. For some responses such as *sn*-2-monoolein or oleic acid, the PFV was not optimal, yet the % of RSD was equal or smaller compared to the original PFV of 1. As a conclusion from this PFV level effect, we concluded to use a PFV value of 1.6 to be employed for all six analytes. By performing this PFV screening experiment, it was possible to maximize the linear dynamic range of all analytes. At the same time, baseline noise was reduced and baseline signal flattened compared to the default PFV (data not shown).

3.2 HPLC-CAD method validation

After optimizing the HPLC gradient program and detector settings, we proceeded with the method validation process by evaluating the performance of the proposed chromatographic method. In this case, we included six validation parameters: limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, and quantitation range. Results of the validation process are compiled in Table 3 for the six analytes evaluated in this study. The LOD and LOQ were determined based on a signal-to-noise ratio of 1:3 and 1:10, respectively. In practice, LOD values ranged from 0.8 to 7.3 ng, and LOQ from 2.7

to 24.4 ng. In another study, LOQ values were determined for fatty acids with different chain lengths using a UPLC-CAD method, but applying reverse phase chromatography (Schilling et al., 2018). These researchers obtained LOQ values ranging from 1.4 to 8.1 ng, which are close to the values obtained for the neutral lipids considered in this study.

The linearity of the calibration curves was assessed after injecting 7 serial dilutions of analytes (Table 3). Calibration curves exhibited a linear behavior in a wide range, going from AOC values between 50 and 3000 ng (up to 60-fold range). We evaluated the linearity of the calibration curves through the calculation of the determination coefficient (R^2), % of the y-intercept, and relative standard deviation (RSD). Calculation of R^2 resulted in values of 0.999 for all cases. The % of the y-intercept varied between 0.5 and 2.6%, and RSD values were below 8.4%. The % of the y-intercept is the ratio between the y-intercept and an a specific amount on column (AOC) as detailed in Table 1. It represents the deviation extent of the y-intercept from the origin in the coordinate plane. This indicator was employed before to evaluate the linearity of calibration curves (Soliven et al., 2017). In addition, we carried out an Analysis of Variance (ANOVA) to prove that the linear regression model was valid for the six analytes. The ANOVA results can be found in the Supplementary material section (Tables S6-S11). All these linearity indicators were in agreement with the acceptance criteria stated in Table 1.

In addition, the method accuracy was tested by estimating the recovery after spiking a known amount of analyte in extraction solution (diethylether:heptane 1:1) at three concentration levels (Table 1). Afterwards, the analyte was quantified using the previously obtained calibration curve. The % of recovery varied between 100 and 104 % for triolein; 102 and 111 % for *sn*-1,3-diolein; 93 and 105 % for *sn*-1,2/2,3-diolein; 96 and 105 % for *sn*-1/3-monoolein; 102 and 114 % for *sn*-2-monoolein; and 97 and 100 % for oleic acid.

In case of precision evaluation, we determined the coefficient of variation (CV) repeated in triplicate, yet in three different concentrations. Results showed low CV values ranging between 0.4 and 1.9 % for triolein; 0.9 and 2.1 % for *sn*-1,3-diolein; 0.7 and 2.2 % for *sn*-1,2/2,3-diolein; 1.1 and 2.1 % for *sn*-1/3-monoolein; 0.5 and 1.1 % for *sn*-2-monoolein; and 0.7 and 2.3 % for oleic acid. All these levels of accuracy and

precision were in accordance with the acceptance criteria defined by FDA and indicated in Table 1 (U.S. Food and Drug Administration, 2018). In the end, the quantitation range was determined by integrating the results of LOQ, linearity, accuracy and precision. These quantitation ranges for all analytes are listed in Table 3.

3.3 HPLC-CAD method application

Once the HPLC-CAD method was established and validated, its potential was tested in the context of evaluation of *in vitro* generated lipid digestion products. First, we prepared an emulsion composed of 5% pure triolein and stabilized with 1% of NaTDC (Section 2.5.1). Second, this emulsion was subjected to *in vitro* digestion conditions so that triolein molecules initially present in the emulsion could be enzymatically converted into *sn*-1,2/2,3-diolein; *sn*-1,3-diolein; *sn*-2-monoolein; *sn*-1/3-monoolein and oleic acid (Section 2.5.2). Third, the remaining triolein and/or generated lipolysis products were extracted. Fourth, all neutral lipids were quantified using the optimized HPLC-CAD method (Section 2.5.3). A chromatogram of a triolein emulsion after 10 min of *in vitro* small intestinal conditions is shown in Fig. 6a. In this chromatogram, all six analytes were identified and could be quantified, yet an extra peak of the lipase inhibitor, Orlistat (retention time 7.2 min), appeared immediately after the oleic acid peak (retention time 6.9 min). After comparing Fig. 6a and Fig. 2c, a slightly earlier elution of oleic acid was observed. We suggest that oleic acid and Orlistat competed for the adsorption to the stationary phase material, leading to a slightly earlier elution of oleic acid in case Orlistat is also present.

From the obtained chromatogram, we calculated the concentration of each analyte as function of digestion time using the corresponding calibration curves and considering all dilution factors. Eventually, we obtained the evolution of each analyte as function of *in vitro* small intestinal digestion (Fig. 6b). There is a clear correlation between triolein hydrolysis, and the production of intermediate products (diolein and monoolein) and the final product (oleic acid). It can even be observed that the generation of *sn*-2-monoolein (empty squares) stopped after 45 min of digestion and started to be cleaved until the end of the simulated digestion process. In order to quantitatively check the correlation between substrate and products, we

performed a mass balance of all six analyte per digestion time (Fig. 6c). The sum of all analytes is similar for the initial emulsion and the digested sample after 5 min of *in vitro* intestinal digestion. This indicates that the applied analytical method reliably quantified triolein and its hydrolysis products. After 5 min of digestion, the total mass concentration slightly decreased and can be explained by the production of glycerol which is not quantified as part of this HPLC-CAD technique. Production of glycerol has been indicated before during *in vitro* digestion studies (Carrière et al., 1997).

4. Conclusion

A reliable and fast HPLC-CAD method was developed and validated for the simultaneous quantification of triolein, *sn*-1,2/2,3-diolein; *sn*-1,3-diolein; *sn*-2-monoolein; *sn*-1/3-monoolein and oleic acid. The final elution gradient program has a duration of 20 min, which permits a relatively high throughput of 72 samples/day. Optimizing the detector settings allowed the maximization and linearity increase of analyte signals. A very high sensitivity of this detector was found during the validation process: analytes could be detected and quantified at a 'nano' level. Linear ranges for the six analytes started from AOC values of 50 ng and covered masses of up to 50 times with respect to this lowest level in the calibration curve. Accuracy and precision tests demonstrated the robustness of the proposed chromatographic method. This HPLC-CAD method showed that analytes could be successfully recovered and quantified during *in vitro* digestion of a triolein emulsion. The development of this HPLC-CAD method is intended to be applied in experiments in which multiple products from triolein hydrolysis need to be quantified. Quantification of multiple lipolysis products is an important element to further understand the kinetics of lipid digestion and the molecular mechanism of these enzymatic reactions. As a future perspective, this method can be a starting point to develop other analytical techniques to simultaneously quantify lipid species produced from commercial oils by including other types of columns, solvents, and/or detectors.

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Declaration of interests

The authors of this work declare no conflict of interests.

References

- Almeling, S., Ilko, D., Holzgrabe, U., 2012. Charged aerosol detection in pharmaceutical analysis. J. Pharm. Biomed. Anal. <https://doi.org/10.1016/j.jpba.2012.03.019>
- Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A.R., Martins, C., Marze, S., McClements, D.J., Ménard, O., Minekus, M., Portmann, R., Santos, C.N., Souchon, I., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Recio, I., 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat. Protoc. 14, 991–1014. <https://doi.org/10.1038/s41596-018-0119-1>
- Carrière, F., Rogalska, E., Cudrey, C., Ferrato, F., Laugier, R., Verger, R., 1997. In vivo and in vitro studies on the stereoselective hydrolysis of tri- and diglycerides by gastric and pancreatic lipases. Bioorg. Med. Chem. 5, 429–435. [https://doi.org/10.1016/S0968-0896\(96\)00251-9](https://doi.org/10.1016/S0968-0896(96)00251-9)
- Couëdelo, L., Amara, S., Lecomte, M., Meugnier, E., Monteil, J., Fonseca, L., Pineau, G., Cansell, M., Carrière, F., Michalski, M.C., Vaysse, C., 2015. Impact of various emulsifiers on ALA

381 bioavailability and chylomicron synthesis through changes in gastrointestinal lipolysis. *Food Funct.*
 382 6, 1726–1735. <https://doi.org/10.1039/C5FO00070J>

383 Fahy, E., Cotter, D., Sud, M., Subramaniam, S., 2011. Lipid classification, structures and tools. *Biochim.*
 384 *Biophys. Acta - Mol. Cell Biol. Lipids* 1811, 637–647. <https://doi.org/10.1016/j.bbalip.2011.06.009>

385 Gamache, P.H., 2017. Charged Aerosol Detection for Liquid Chromatography and Related Separation
 386 Techniques, Charged Aerosol Detection for Liquid Chromatography and Related Separation
 387 Techniques. John Wiley & Sons, Inc., Hoboken, NJ, USA. <https://doi.org/10.1002/9781119390725>

388 Górecki, T., Lynen, F., Szucs, R., Sandra, P., 2006. Universal response in liquid chromatography using
 389 charged aerosol detection. *Anal. Chem.* 78, 3186–3192. <https://doi.org/10.1021/ac060078j>

390 Graeve, M., Janssen, D., 2009. Improved separation and quantification of neutral and polar lipid classes
 391 by HPLC–ELSD using a monolithic silica phase: Application to exceptional marine lipids. *J.*
 392 *Chromatogr. B* 877, 1815–1819. <https://doi.org/10.1016/j.jchromb.2009.05.004>

393 Haidar Ahmad, I.A., Blasko, A., Tam, J., Variankaval, N., Halsey, H.M., Hartman, R., Regalado, E.L.,
 394 2019. Revealing the inner workings of the power function algorithm in Charged Aerosol Detection:
 395 A simple and effective approach to optimizing power function value for quantitative analysis. *J.*
 396 *Chromatogr. A* 1603, 1–7. <https://doi.org/10.1016/j.chroma.2019.04.017>

397 Hasan, F., Shah, A.A., Hameed, A., 2009. Methods for detection and characterization of lipases: A
 398 comprehensive review. *Biotechnol. Adv.* 27, 782–798.
 399 <https://doi.org/10.1016/j.biotechadv.2009.06.001>

400 Hutchinson, J.P., Li, J., Farrell, W., Groeber, E., Szucs, R., Dicinoski, G., Haddad, P.R., 2011.
 401 Comparison of the response of four aerosol detectors used with ultra high pressure liquid
 402 chromatography. *J. Chromatogr. A* 1218, 1646–1655. <https://doi.org/10.1016/j.chroma.2011.01.062>

403 Infantes-Garcia, M.R., Verkempinck, S.H.E., Guevara-Zambrano, J.M., Andreoletti, C., Hendrickx, M.E.,

- Grauwet, T., 2020. Enzymatic and chemical conversions taking place during in vitro gastric lipid digestion: The effect of emulsion droplet size behavior. *Food Chem.* 326, 126895.
<https://doi.org/10.1016/j.foodchem.2020.126895>
- Karupaiah, T., Sundram, K., 2007. Effects of stereospecific positioning of fatty acids in triacylglycerol structures in native and randomized fats: a review of their nutritional implications. *Nutr. Metab. (Lond)*. 4, 16. <https://doi.org/10.1186/1743-7075-4-16>
- Li, S., Tian, M., Row, K.H., 2010. Effect of Mobile Phase Additives on the Resolution of Four Bioactive Compounds by RP-HPLC. *Int. J. Mol. Sci.* 11, 2229–2240. <https://doi.org/10.3390/ijms11052229>
- Lucas-González, R., Viuda-Martos, M., Pérez-Alvarez, J.A., Fernández-López, J., 2018. In vitro digestion models suitable for foods: Opportunities for new fields of application and challenges. *Food Res. Int.* <https://doi.org/10.1016/j.foodres.2018.02.055>
- McClements, D.J., Li, Y., 2010. Review of in vitro digestion models for rapid screening of emulsion-based systems. *Food Funct.* 1, 32. <https://doi.org/10.1039/c0fo00111b>
- Miller, W.M., Nori-Janosz, K.E., Lillystone, M., Yanez, J., McCullough, P.A., 2005. Obesity and lipids. *Curr. Cardiol. Rep.* 7, 465–470. <https://doi.org/10.1007/s11886-005-0065-8>
- Moreau, R.A., 2006. The analysis of lipids via HPLC with a charged aerosol detector. *Lipids* 41, 727–734. <https://doi.org/10.1007/s11745-006-5024-7>
- Schilling, K., Holzgrabe, U., 2020. Recent applications of the Charged Aerosol Detector for liquid chromatography in drug quality control. *J. Chromatogr. A* 1619, 460911.
<https://doi.org/10.1016/j.chroma.2020.460911>
- Schilling, K., Pawellek, R., Lovejoy, K., Muellner, T., Holzgrabe, U., 2018. Influence of charged aerosol detector instrument settings on the ultra-high-performance liquid chromatography analysis of fatty acids in polysorbate 80. *J. Chromatogr. A* 1576, 58–66.

427 <https://doi.org/10.1016/j.chroma.2018.09.031>

428 Soliven, A., Haidar Ahmad, I.A., Tam, J., Kadrichu, N., Challoner, P., Markovich, R., Blasko, A., 2017.

429 A simplified guide for charged aerosol detection of non-chromophoric compounds—Analytical
430 method development and validation for the HPLC assay of aerosol particle size distribution for
431 amikacin. *J. Pharm. Biomed. Anal.* 143, 68–76. <https://doi.org/10.1016/j.jpba.2017.05.013>

432 U.S. Environmental Protection Agency, 2021. CompTox Chemicals Dashboard [WWW Document]. URL
433 <https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID1025809#properties> (accessed
434 3.23.21).

435 U.S. Food and Drug Administration, 2018. Bioanalytical Method Validation Guidance for Industry
436 Biopharmaceutics Bioanalytical Method Validation Guidance for Industry.

437 Verkempinck, S.H.E., Salvia-Trujillo, L., Moens, L.G., Charleer, L., Van Loey, A.M., Hendrickx, M.E.,
438 Grauwet, T., 2018. Emulsion stability during gastrointestinal conditions effects lipid digestion
439 kinetics. *Food Chem.* 246, 179–191. <https://doi.org/10.1016/J.FOODCHEM.2017.11.001>

440 Yaqoob, P., 2013. Role of Lipids in Human Nutrition, in: *Handbook of Olive Oil*. Springer US, Boston,
441 MA, pp. 655–675. https://doi.org/10.1007/978-1-4614-7777-8_17

Caption of Figures

Figure 1. Gradient elution programs implemented in this research. (a) Graeve & Janssen (2009) program, (b-e) representative examples of modified programs tested in the present work, and (f) the final elution program obtained during the HPLC-CAD optimization process. Solid lines are related to the primary y-axis (left side) and represent the evolution % of solvent A (blue), B (green), C (grey), and D (yellow), while the dashed line represents the flow rate evolution during the gradient program (secondary y-axis, right side).

Figure 2. (a) HPLC-CAD chromatograms after blank injections using different gradient programs. HPLC-CAD chromatograms after injecting a mixture of neutral lipid standards using the (b) elution program implemented by Graeve & Janssen (2009), and (c) the final elution program developed in the present work. For graph (a), roman numbers denote the (I) elution program implemented by Graeve & Janssen (2009) (corresponds to elution program in Figure 1a), modified elution programs corresponding to (II) Figure 1b, (III) 1d, (IV) 1e and (V) 1f (final elution program) tested in the present work. For graphs (b) and (c), numbers on top of peaks indicate analytes corresponding to: (1) triolein, (2) mixture of diolein isomers, (2') *sn*-1,3-diolein, (2'') *sn*-1,2/2,3-diolein, (3) oleic acid, (4) mixture of monoolein isomers, (4') *sn*-1/3-monoolein, and (4'') *sn*-2-monoolein.

Figure 3. Comparison of the individual and total peak areas obtained after injecting neutral lipid standards to the HPLC-CAD system using the two evaporator temperatures that can be set in the detector.

Figure 4. Response factor (peak area/AOC) as a function of AOC (ng) levels determined for neutral lipids. Symbols in graphs correspond to data collected using different power function values (PFV): (○) PFV 1, (◆) PFV 1.2, (■) PFV 1.4, (▲) PFV 1.6, and (●) PFV 1.8. Lines connecting data points do not represent model lines, but are added to facilitate graph reading.

Figure 5. Variation of the relative standard deviation (% RSD) of the response factor linearity depending on the power function value (PFV) determined for six neutral lipids.

Figure 6. (a) HPLC-CAD chromatogram of an extract from a partially digested triolein emulsion after 10 min of *in vitro* small intestinal digestion. (b) Evolution of triolein and its hydrolysis products during *in vitro* small intestinal digestion of a 5% triolein emulsion stabilized with 1% of sodium taurodeoxycholate. (c) Comparison between the

total mass concentration of analytes quantified for the initial emulsion and samples digested during *in vitro* small intestinal phase (SIP). For graph (a), numbers on top of peaks indicate analytes corresponding to: (1) triolein, (2) *sn*-1,3-diolein, (3) *sn*-1,2/2,3-diolein, (4) oleic acid, (5) lipase inhibitor (Orlistat), (6) *sn*-1/3-monoolein, and (7) *sn*-2-monoolein.

Caption of Tables

Table 1. Parameters, methodology and acceptance criteria employed to validate the HPLC-CAD chromatographic method to quantify triolein and its hydrolysis products (lipid standards) based on FDA criteria.

Table 2. Final gradient elution program obtained after HPLC-CAD method optimization. Solvent A: isooctane; solvent B: acetone:ethyl acetate (2:1) containing 0.02% acetic acid ; solvent C: 2-propanol:water (85:15) containing 0.05% acetic acid & 0.05% ethanolamine; and solvent D: 2-propanol.

Table 3. Results of the chromatographic method validation process for triolein and its hydrolysis products (lipid standards).