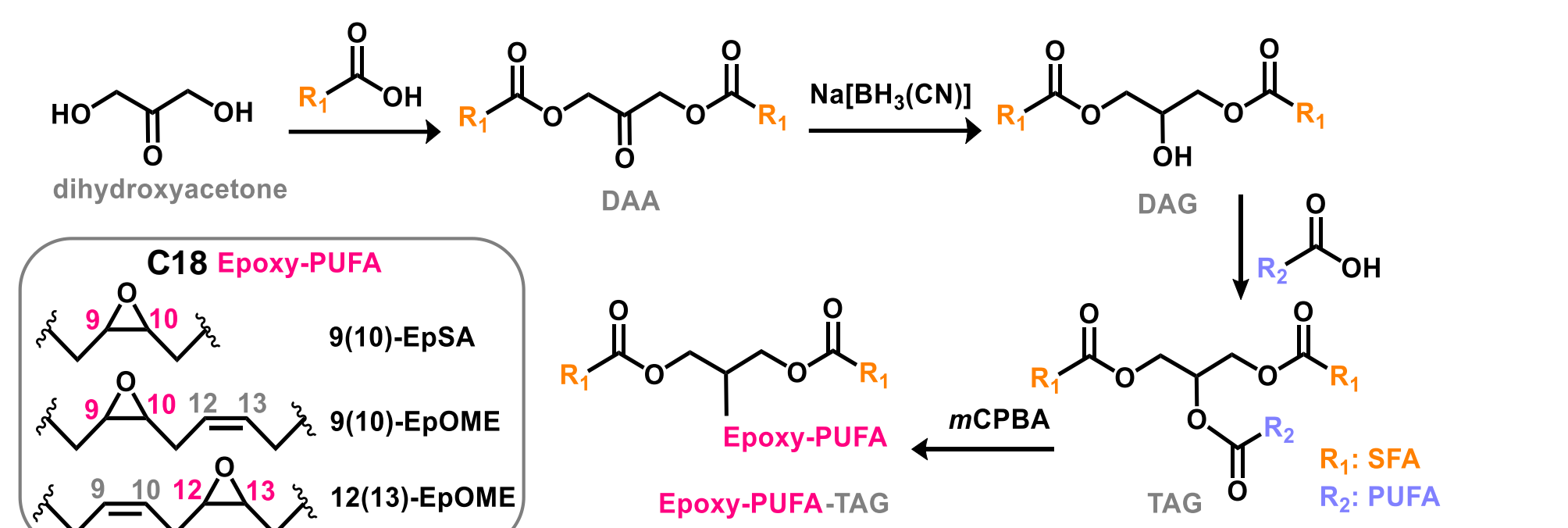


## Introduction

Polyunsaturated fatty acids (PUFA) are susceptible to oxidation, leading to the formation of various oxidation products, known as oxylipins, such as hydro(pero)xy-PUFA and epoxy-PUFA. Epoxy-PUFA occur in edible oils and are formed e.g. during deep frying [1,2]. Endogenously formed epoxy-PUFA are lipid mediators regulating inflammatory processes and vascular tone. While biological effects of epoxy-PUFA have primarily been described for free epoxy-PUFA, they occur - as other oxylipins - in biological samples esterified to polar lipids like glycerophospholipids and neutral lipids such as triacylglycerols (TAG). Currently, esterified epoxy-PUFA in biological samples are indirectly quantified following saponification by targeted liquid chromatography mass spectrometry (LC-MS) of the resulting free oxylipins [3]. However, the information about the lipid class in which the epoxy-PUFA are bound is lost. In this study, TAG bearing epoxy-PUFA occurring in vegetable oils and human cell lines were characterized using synthetic standards and reversed-phase liquid chromatography high-resolution MS (LC-HRMS).

## Synthesis of TAG16:0/epoxy-PUFA/16:0



**Fig. 1: Epoxy-PUFA-TAG synthesis:** Steglich esterification of dihydroxyacetone with SFA using 4-Dimethylaminopyridine (DMAP) and dicyclohexylcarbodiimide (DCC), reduction of diacylacetone (DAA) with  $\text{Na}[\text{BH}_2(\text{CN})]$  and Steglich esterification of diacylglycerol (DAG) with a PUFA using DMAP and DCC. Epoxidation of double bond with *meta*-chloroperoxybenzoic acid (*m*CPBA) yielding a *sn*-2-epoxy-PUFA-TAG.

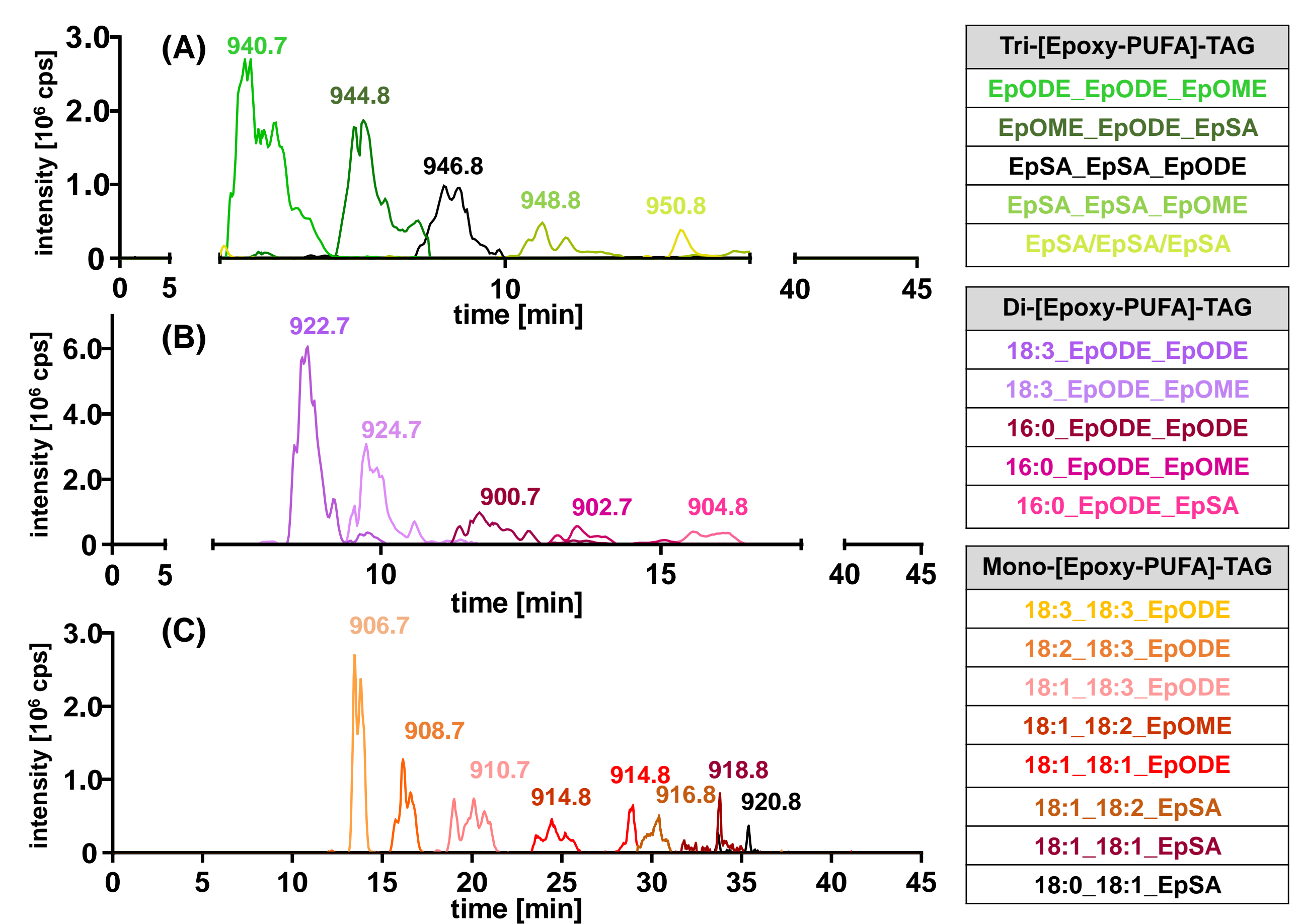
## LC-HRMS characterization of epoxy-PUFA-TAG in linseed oil treated with *m*CPBA

- To generate a large variety of potentially occurring epoxy-PUFA-TAG, linseed oil, containing 56% 18:3, 14% 18:2 and 19% 18:1, was chemically epoxidized using *m*CPBA. Yielding epoxy-PUFA-TAG were compared to synthesized epoxy-PUFA-TAG standards (Fig.1-2).

- Several peaks of epoxy-PUFA TAG were found in LC-HRMS analysis (Fig.3).

- Based on the fragmentation pattern of epoxy-PUFA-TAG (Fig.2) the peaks could be characterized as TAG bearing epoxy derivatives of oleic acid (EpSA), linoleic acid (EpOME) and  $\alpha$ -linolenic acid (EpODE).

- Epoxy-PUFA-TAG bearing one (Mono-[Epoxy-PUFA]-TAG) two (Di-[Epoxy-PUFA]-TAG) and three (Tri-[Epoxy-PUFA]-TAG) were found with a characteristic elution pattern.



**Fig. 3:** RP-LC-ESI(+)-HRMS analysis of epoxidized linseed oil. Linseed oil was epoxidized using *m*CPBA and epoxy-PUFA-TAG were detected as ammonium adducts ( $[\text{M}+\text{NH}_4]^+$ ). Shown are extracted ion chromatograms (XIC) of most abundant (A) Tri-[Epoxy-PUFA]-TAG, (B) Di-[Epoxy-PUFA]-TAG, and (C) Mono-[Epoxy-PUFA]-TAG species, characterized based on specific fragments (Fig. 2). The analysis was focused on fatty acids occurring in linseed oil: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2),  $\alpha$ -linolenic acid (18:3) and the epoxy derivatives of oleic acid (EpSA), linoleic acid (EpOME) and  $\alpha$ -linolenic acid (EpODE).

→ LC-HRMS analysis of the epoxidized linseed oil demonstrates the large number of possible epoxy-PUFA-TAG species which were well separated by RP-LC-MS and characterized based on specific fragment ions (Fig 2-3).

## LC-HRMS characterization of epoxy-PUFA-TAG formed in rapeseed oil during deep frying

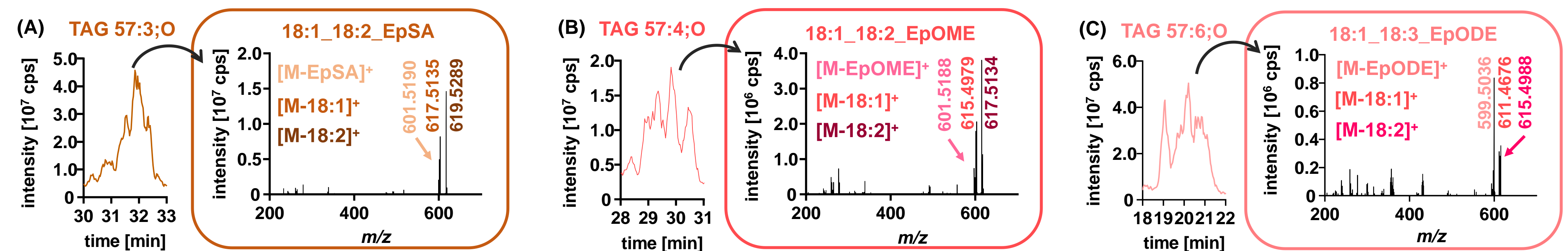
- Rapeseed oil was used for 20 frying cycles, 3 min each at 175 ° [1]

→ Epoxy-PUFA increase in rapeseed oil during deep frying. [1,2]

- Epoxy-PUFA-TAG were characterized by LC-HRMS:

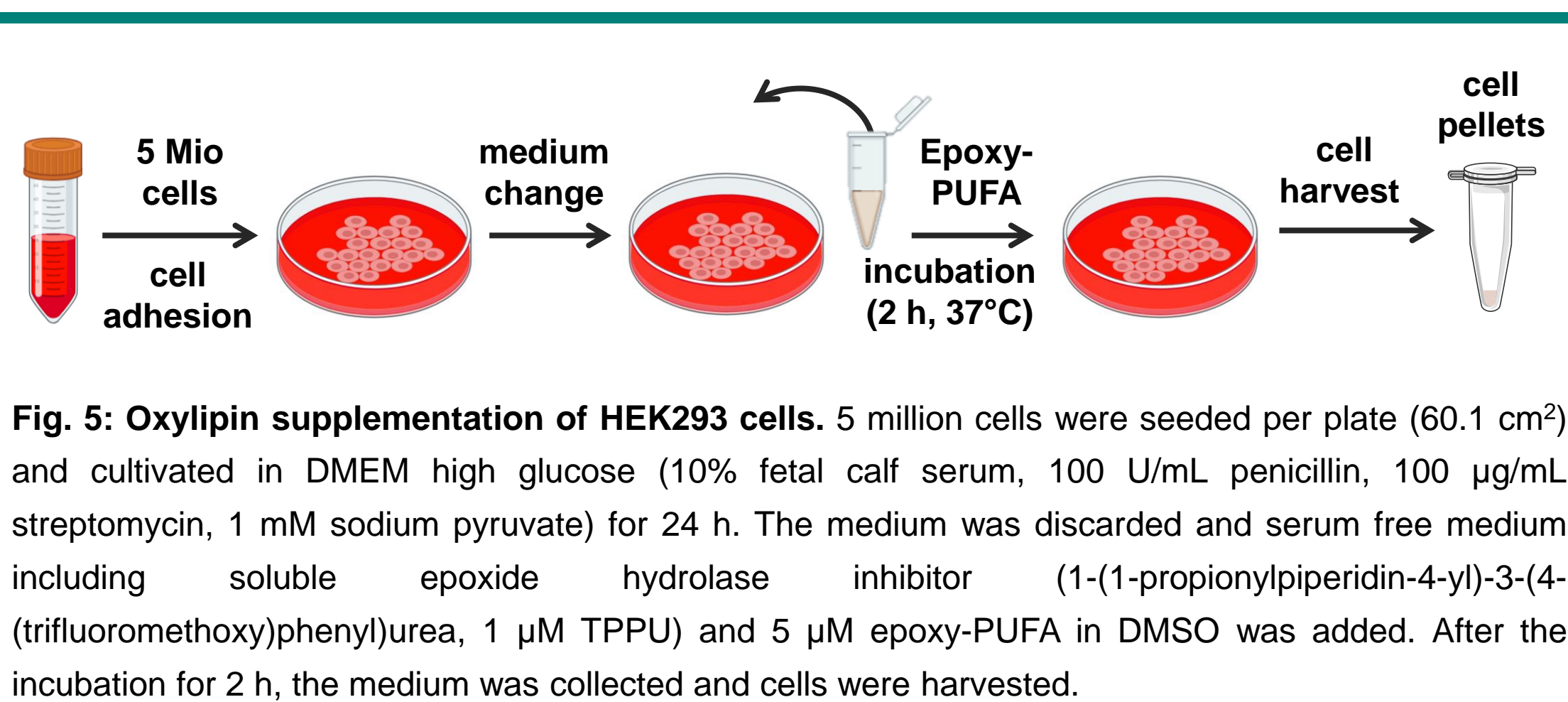
- Mono-[Epoxy-PUFA]-TAG were the most abundant epoxy-PUFA-TAG characterized, based on specific fragment ions (Fig 2-3).

- Deep frying leads to epoxy-PUFA-TAG, bearing EpSA, EpOME and EpODE, esterified together with PUFA such as 18:1, 18:2 and 18:3 which are the main fatty acids in rapeseed oil.

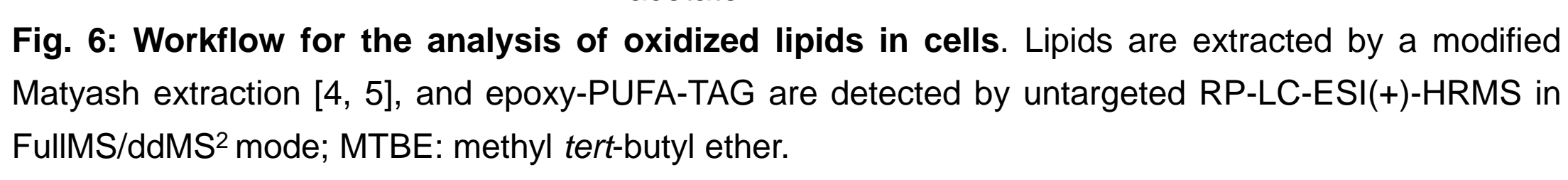


**Fig. 4:** RP-LC-ESI(+)-HRMS analysis of oxidized TAG in rapeseed oil repeatedly used for deep frying. Shown are the XIC of  $[\text{M}+\text{NH}_4]^+$  ions at  $m/z$  916.7964 (TAG 57:3:0) (A)  $m/z$  914.7807 (TAG 57:4:0) (B) and  $m/z$  910.7495 (TAG 57:6:0) (C), together with corresponding fragment spectra. Structural characterization of the epoxy-PUFA-TAG was carried out based on the characteristic fragmentation pattern (Fig.2).

## LC-HRMS characterization of epoxy-PUFA-TAG generated by HEK293 cells following supplementation

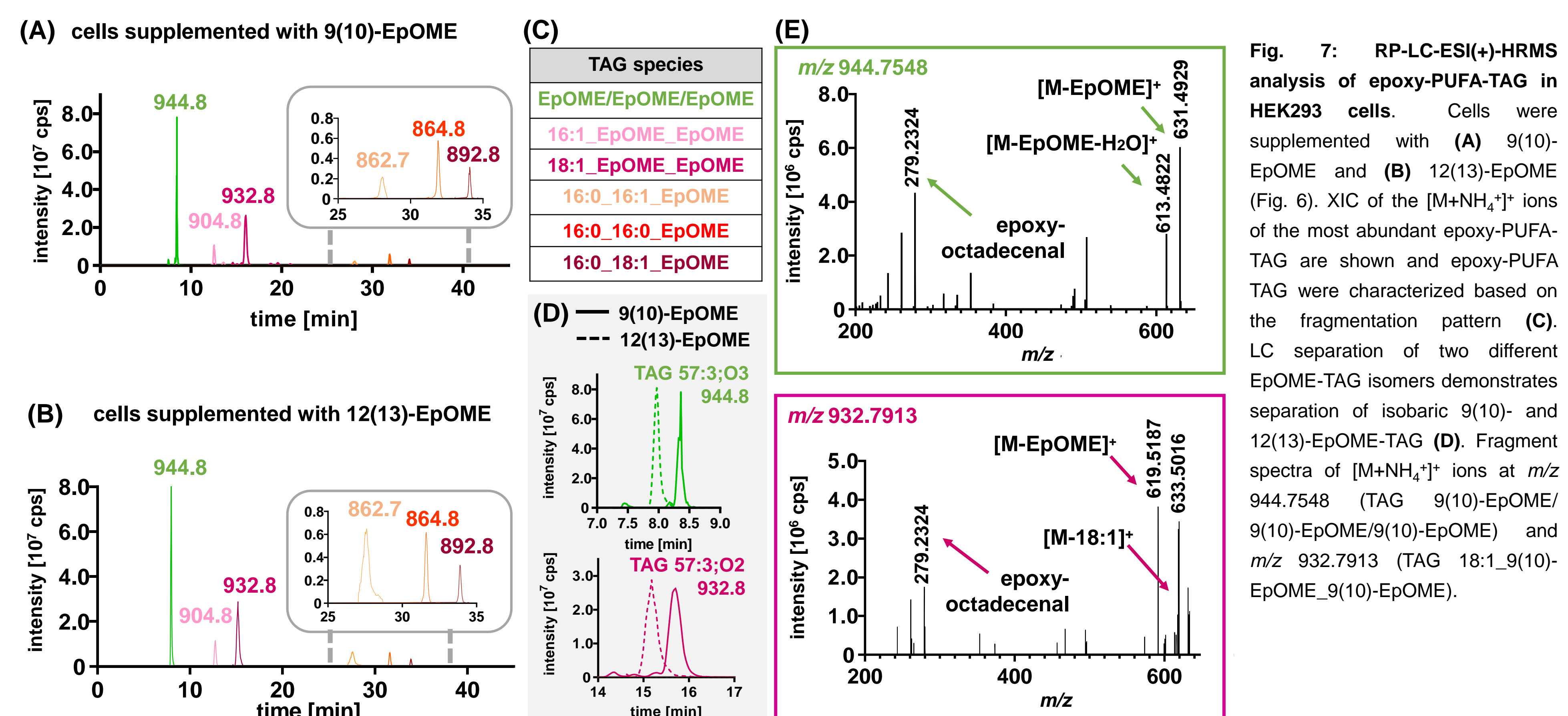


**Fig. 5: Oxylin supplementation of HEK293 cells.** 5 million cells were seeded per plate (60.1 cm<sup>2</sup>) and cultivated in DMEM high glucose (10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate) for 24 h. The medium was discarded and serum free medium including soluble epoxide hydrolase inhibitor (1-(1-propionylpiperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea, 1 µM TPPU) and 5 µM epoxy-PUFA in DMSO was added. After the incubation for 2 h, the medium was collected and cells were harvested.



- Following supplementation of HEK293 cells (Fig. 5-6) both 9(10)-EpOME and 12(13)-EpOME were partially esterified into TAG.

- Both EpOME isomers are similarly absorbed and esterified by the cells, leading to the formation of characteristic epoxy-PUFA-TAG species (Fig. 7).



**Fig. 7:** RP-LC-ESI(+)-HRMS analysis of epoxy-PUFA-TAG in HEK293 cells. Cells were supplemented with (A) 9(10)-EpOME and (B) 12(13)-EpOME (Fig. 6). XIC of the  $[\text{M}+\text{NH}_4]^+$  ions of the most abundant epoxy-PUFA-TAG are shown and epoxy-PUFA TAG were characterized based on the fragmentation pattern (C). LC separation of two different EpOME-TAG isomers demonstrates separation of isobaric 9(10)- and 12(13)-EpOME-TAG (D). Fragment spectra of  $[\text{M}+\text{NH}_4]^+$  ions at  $m/z$  944.7548 (TAG 9(10)-EpOME/9(10)-EpOME/9(10)-EpOME) and  $m/z$  932.7913 (TAG 18:1\_9(10)-EpOME\_9(10)-EpOME).

## Literature

[1] Koch, E., et al., Trans-Hydroxy, Trans-Epoxy, and Erythro-dihydroxy Fatty Acids Increase during Deep-Frying. Journal of agricultural and food chemistry, 2023, 71(19): p. 7508-7513  
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## Conclusion

A large number of epoxy-PUFA-TAG species are formed in edible oil during deep frying and in human cells following supplementation. This includes Mono-, Di- and Tri-[Epoxy-PUFA]-TAG bearing EpSA, EpOME and EpODE as well as the major FA in the samples. Chemical synthesis of standards and epoxygenation of vegetable oils allows to generate standard (mixture)s enabling the characterization/identification of the epoxy-PUFA-TAG species.