Targeted Isolation of 5,6-Epoxy, 7-Keto, and 7-OH Phytosterol Oxidation Products via semipreparative LC-PDA-MS

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INTRODUCTION

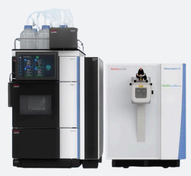
Phytosterols are bioactive plant-derived compounds widely present in vegetable oils and known for their beneficial effects on human health[1]. However, due to their unsaturated structures, phytosterols are **prone to oxidation**, leading to the formation of **phytosterol oxidation products** (POPs), that may negatively impact health and oil quality[2]. Mass spectrometry is widely used to analyze POPs, but the **lack** of commercially available pure POP standards (with the exception of 7-ketositosterol) **limits method accuracy**. Thus, reliable detection of these compounds is challenging due to the lack of pure standards and limitations of conventional methods[3].

The aim of the present work was to **isolate pure POPs** (α-epoxy, β-epoxy, 7-keto, 7α- and 7β-hydroxy isomers of β-sitosterol, campesterol, and stigmasterol) using a semi-preparative LC system, and to **validate** a robust, sensitive, precise, and accurate LC-Orbitrap-HRMS method.

MATERIALS AND METHODS

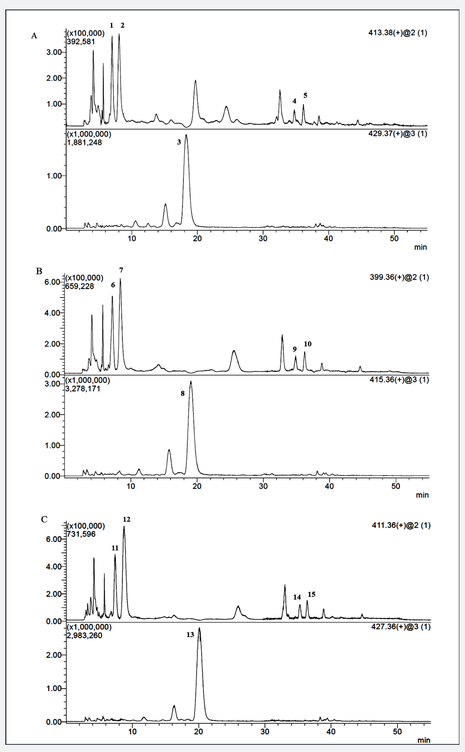
- **Oxidation** of phytosterols (180°C; 90 min)

- A customized semi-preparative HPLC system was used to isolate and **collect the targeted POPs**.



- The composition of each **collected fraction** was evaluated by HPLC-HRMS Orbitrap® and GC–MS, while the quantification and purity by GC-FID was carried out.
- Linearity, sensitivity, precision, and accuracy was assessed according to international guidelines.
- **Medium chain triacylglycerols** (MCT) were used as a blank reference matrix to prepare calibration curves and to perform recovery experiments.

RESULTS



1= 5,6α-epoxysitosterol
2= 5,6β-epoxysitosterol
3= 7-ketositosterol
4= 7α-hydroxsitosterol
5= 7β-hydroxysitosterol

6= 5,6α-epoxycampesterol
7= 5,6β-epoxycampesterol
8= 7-ketocampesterol
9= 7α-hydroxycampesterol
10= 7β-hydroxycampesterol

11= 5,6α-epoxystigmasterol
12= 5,6β-epoxystigmasterol
13= 7-ketostigmasterol
14= 7α-hydroxystigmasterol
15= 7β-hydroxystigmasterol

POPs	RT (min)	Precursor ions (*m/z*) $M+H^+$	Product ions (*m/z*) $M-H_2O+H^+$	Product ions (*m/z*) $M-2H_2O+H^+$
β-Sitosterol				
α-Es	12.93	431.3890 (–)	413.3785 **(33)**	395.3679 **(100)**
β-Es	16.61	431.3895 (–)	413.3792 **(25)**	395.3683 **(100)**
7-Ks	26.01	429.3740 (100)	411.3624 **(45)**	393.3521 **(24)**
7α-Hs	29.63	431.3813 (–)	413.3797 **(15)**	395.3685 **(100)**
7β-Hs	30.38	431.3533 (–)	413.3790 **(12)**	395.3682 **(100)**
Campesterol				
α-Ec	15.03	417.3744 (–)	399.3638 **(45)**	381.3532 **(100)**
β-Ec	16.95	417.3745 (–)	399.3634 **(43)**	381.3539 **(100)**
7-Kc	26.22	415.3578 **(100)**	397.3474 **(45)**	379.3367 **(24)**
7α-Hc	29.77	417.3380 (–)	399.3636 **(15)**	381.3526 **(100)**
7β-Hc	30.53	417.3654 (–)	399.3627 **(26)**	381.3522 **(100)**
Stigmasterol				
α-Est	15.04	429.3737 (–)	411.3633 **(80)**	393.3524 **(100)**
β-Est	16.90	429.3735 (–)	411.3630 **(100)**	393.3523 **(75)**
7-Kst	26.26	427.3578 **(100)**	409.3473 **(45)**	391.3366 **(24)**
7α-Hst	29.77	429.3649 (–)	411.3628 (45)	393.3523 **(100)**
7β-Hst	30.42	429.3645 (–)	411.3626 (50)	393.3521 **(100)**

Abbreviations: 7-K, 7-keto sterol; 7α-H, 7α-hydroxy sterol; 7β-H, 7β-hydroxy sterol; s, sitosterol, c, campesterol; st, stigmasterol; α-E, α-epoxy sterol; β-E, β-epoxy sterol.

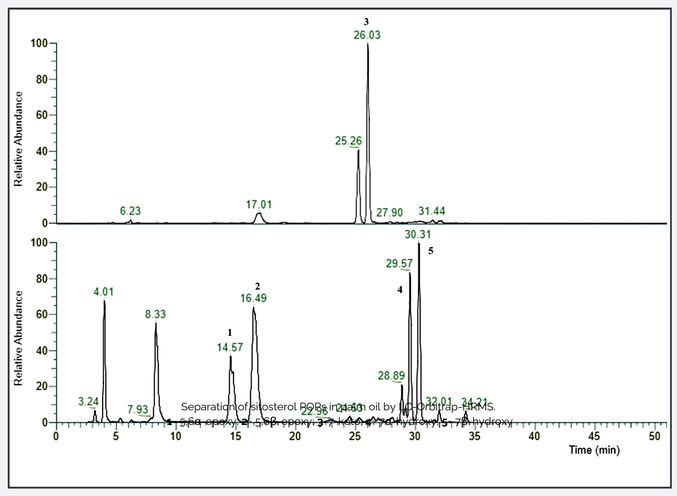
Sitosterol, **campesterol**, and **stigmasterol** displayed a similar fragmentation behavior with unique differences in their relative abundance of ions.

The **7-keto** derivates displayed the protonated molecular ion $[M + H]^+$ as the base peak and showed a loss of one and two water molecules only after the MS^2 fragmentation.

The **LOD** and **LOQ** confirmed a higher sensitivity of LC-Orbitrap-HRMS with respect to data obtained by GC-FID, GC/MS, or LC–MS/MS.

Under the analytical conditions, the **normal phase** LC method led to **properly determining** α-epoxy, β-epoxy, 7-keto, 7α-hydroxy, and 7β-hydroxy isomers of sterols.

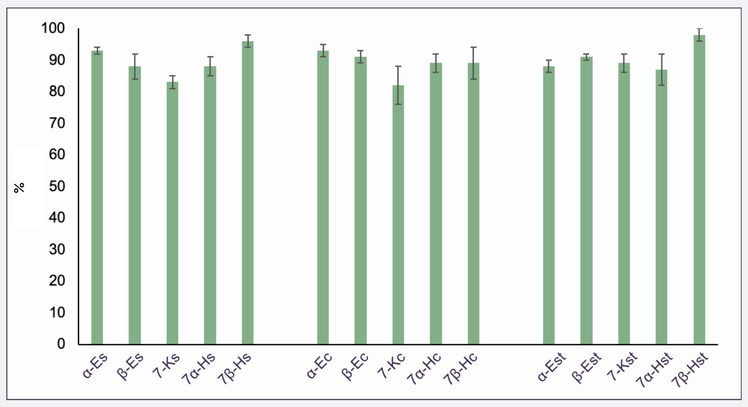
The separation of oxidation products was achieved in less than **40 min** and retention times in both standard mixtures and real samples were **reproducible**.



♦ Purity grade (%) of isolated POPs

Purity (%)	5,6α-E	5,6β-E	7-K	7α-H	7β-H
β-Sitosterol	93.5	96.7	94.3	91.7	91.5
Campesterol	90.2	93.1	92.5	96.3	94.8
Stigmasterol	92.7	91.4	95.3	97.5	91.2

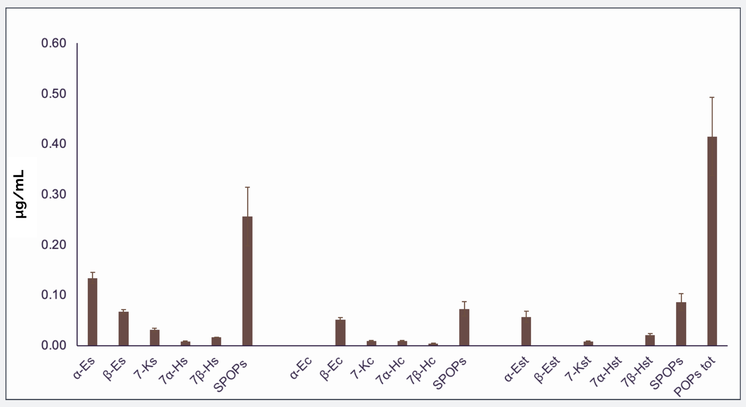
♦ Recovery (%)



The suitability of the developed method was tested in **fresh refined palm oil**.

The total content of POPs was equal to 0.42 µg/mL oil, where the oxidized isomers of β-sitosterol (0.26 µg/mL oil) were the main POPs determined.

The α-epoxy isomers were the main POPs determined for β-sitosterol and stigmasterol.



CONCLUSIONS

- A semi-preparative LC method was successfully **developed to isolate pure POPs** from β-sitosterol, campesterol, and stigmasterol[4].
- **Fifteen** different POPs (α-epoxy, β-epoxy, 7-keto, 7α-OH, 7β-OH derivatives of β-sitosterol, campesterol, and stigmasterol) were isolated in less than 50 minutes, with **purity >90%**.
- The validated LC-Orbitrap-HRMS method proved to be **sensitive**, **precise**, and **robust**, allowing accurate POPs determination **without derivatization** or SPE **purification.**
- The **LOD** and **LOQ** determined in MCT were >0.012 and >0.039 ng/mL, respectively; whereas the recoveries ranged between 82% and 98%.

REFERENCES

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