Research Article

Solanum sessiliflorum (mana-cubiu) antioxidant protective effect towards cholesterol oxidation: influence of docosahexaenoic acid

Blanca Barriuso[1], Lilian Regina Barros Mariutti [2], Diana Ansorena[1*], Iciar Astiasarán[1], Neura Bragagnolo[2]

[1] Department of Nutrition, Food Science and Physiology, Faculty of Pharmacy, University of Navarra, C/ Irunlarrea s/n, 31008. IDISNA- Instituto de Investigación Sanitaria de Navarra, Pamplona, Spain
bbarriuso@alumni.unav.es; iastiasa@unav.es

[2] Department of Food Science, Faculty of Food Engineering,
University of Campinas, Rúa Monteiro Lobato 80, Campinas (São Paulo), Brazil
lilianmariutti@gmail.com; neurabragagnolo@gmail.com

***Correspondence:** email: dansorena@unav.es; telephone: 0034-948425600 (ext. 6263); Fax: +34 948 42 56 49.

Running Title: *Mana-cubiu* protective effect towards cholesterol oxidation

Keywords: oxysterols, docosahexaenoic acid, oxidation, natural antioxidants

Abbreviations: COPs, cholesterol oxidation products; DHA, docosahexaenoic acid; MCE, mana-cubiu extract

European Journal of Lipid Science and Technology, 118(8), 1125-1131.(2016).

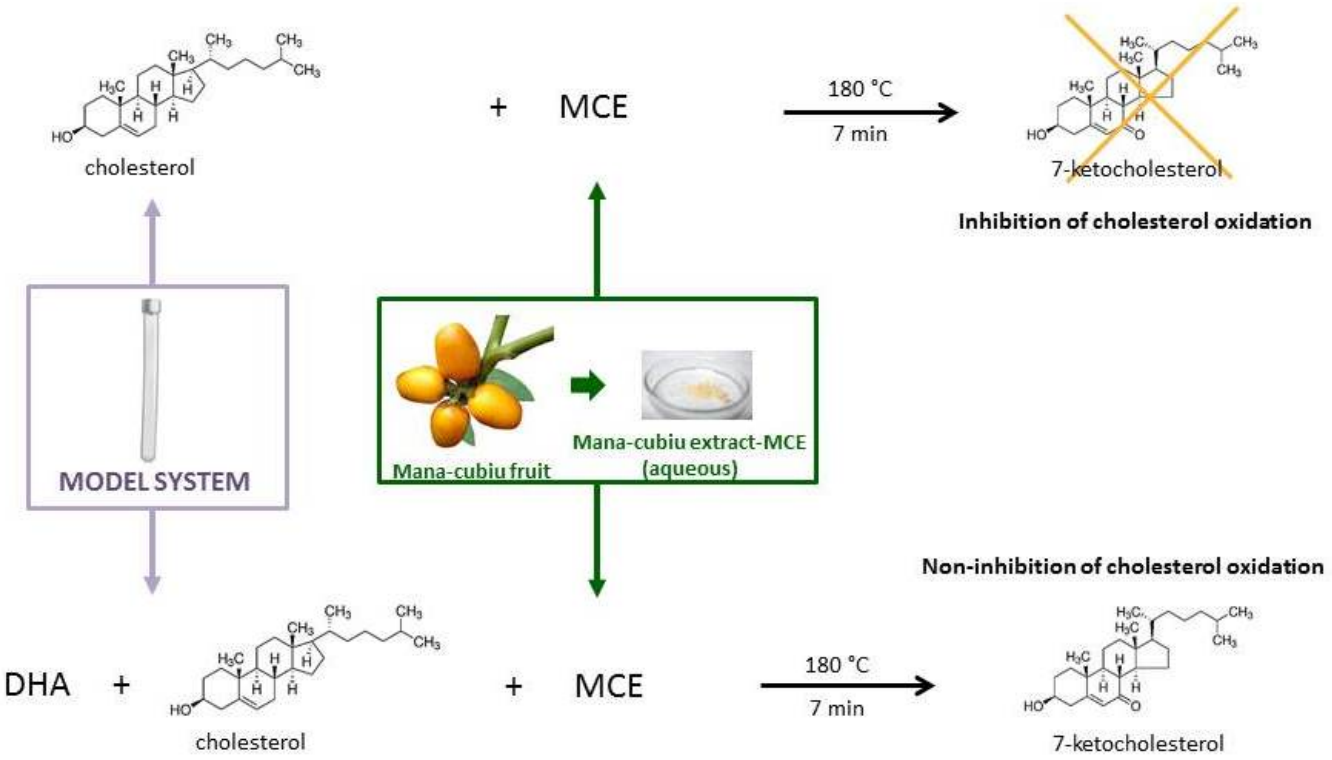
Abstract

Harmful health effects have been attributed to cholesterol oxidation products (COPs). Factors that modulate their formation in foods are light, oxygen, heat, and food matrix (such as antioxidants content or unsaturation degree of lipids), among others. The objective of this work was to assess the effectiveness of an extract obtained from *Solanum sessiliflorum* (mana-cubiu) (MCE) as a potential inhibitor of cholesterol oxidation under heating conditions. The influence of free DHA presence in the system was also evaluated. Results showed that MCE inhibited cholesterol degradation (44 % *vs* 18 % without and with MCE, respectively) and reduced 9-fold COPs formation in the absence of DHA. However, when DHA was present, the MCE was not effective towards cholesterol oxidation. In this case, MCE showed its antioxidant effect protecting DHA from degradation (89 % *vs* 64 %).

Practical applications

Antioxidant properties of this solvent free natural extract make MCE a potential good ingredient in food products containing highly polyunsaturated lipids to protect them from oxidation and in food products lacking polyunsaturated lipids to protect cholesterol from oxidation.

Graphical abstract



Mana-cubiu inhibits cholesterol oxidation in absence of DHA.

1. Introduction

Cholesterol chemical structure makes it an easy-to-oxidize molecule, leading to the formation of oxysterols. These oxidation products, usually named as Cholesterol Oxidation Products (COPs), have been related to several diseases (atherosclerosis, neurodegenerative diseases, mutagenic and carcinogenic effects, etc) [1-3]. They have been found in a variety of animal food samples [1], and some studies have pointed out their potential absorption through the diet [4-5]. Therefore, minimizing their formation during food manufacturing, processing and / or cooking is of great interest to reduce health risk. Use of additives or oxygen-restriction methods is commonly applied for that purpose.

In this sense, a growing interest in natural antioxidants found in plants is noticed, not only because of technological reasons, but also due to their potential ability to suppress oxidative stress and related diseases [6]. Regarding their usefulness in food systems, successful cases in controlling cholesterol oxidation have been reported in several types of matrices [7-10]. Mana-cubiu (*Solanum sessiliflorum*) is a fruit native to

Amazonia, which possess antioxidant properties attributed to the presence of carotenoids and phenolic compounds in its composition [11].

On the other hand, the interest in highly unsaturated fatty acids has recently increased due to their health-related properties. Particularly, long-chain omega-3 polyunsaturated fatty acids have demonstrated cardiovascular disease lowering effects [12-13]. Nevertheless, inadequate manufacturing and cooking conditions can lead to some loss in the content of these interesting compounds. A number of studies have dealt with the prevention of fatty acid degradation through antioxidant addition, after different cooking and storage conditions [8, 14-16]. A possible interaction between cholesterol oxidation and the surrounding fatty acids has been proposed by several authors as a factor that modulates cholesterol oxidation susceptibility, although no consensus on the subject has been found [17-20].

Foods are usually complex matrices where interferences among several components may hamper a clear view about the mechanisms of cholesterol oxidation. Therefore, model systems are a very useful tool to evaluate separately the factors that exert an influence in this process. A variety of antioxidants [19, 21-24], and lipid matrices [20, 25, 26] have been tested in model systems.

Considering the exposed above, the aim of this study was to evaluate the antioxidant protective effect of a *Solanum sessiliflorum* lyophilized food-grade aqueous extract against cholesterol degradation and cholesterol oxidation products formation in a model system, with and without the presence of docosahexaenoic acid (DHA). Furthermore, the effect of refrigeration storage was also evaluated.

2. Material and methods

2.1 Material and reagents

Mana-cubiu fruits (~ 21 Kg) were acquired at CEAGESP (São Paulo General Warehousing and Centers Company, São Paulo, Brazil). Cholesterol, 22R-hydroxycholesterol, 22S-hydroxycholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, 5,6β-epoycholesterol, 5,6α-epoxycholesterol, 7-ketocholesterol and DHA standards were purchased from Sigma-Aldrich (Newport, RI, USA). 7α-hydroxycholesterol and 7β-hydroxycholesterol were purchased from Steraloids (Newport, RI, USA). The purity of the standards was at least 95% as determined by HPLC or GC analyses. Chloroform and methanol were purchased from Synth

(Diadema, SP, Brazil). Chromatographic grade hexane (minimum 63% n-hexane) and 2-propanol were purchased from Panreac (Alvorada, RS, Brazil).

2.2 Mana-cubiu extract preparation and characterization

Mana-cubiu fruits were lyophilized before extraction [11]. Fifty grams of lyophilized mana-cubiu fruit were homogenized with ultrapure water in a vortex for 5 min and centrifuged at 20000 g at 10 °C. The aqueous layer was lyophilized during 120 h at -92 °C below 40 µHg (Liobras, São Paulo, Brazil). The identification and quantification of the phenolic compounds of the mana-cubiu extract (MCE) was carried out according to Rodrigues et al. [11].

2.3 Sample preparation and heating

Stock solutions of cholesterol (1 mg/mL in chloroform), DHA (1 mg/mL in chloroform) and MCE (2.5 mg/mL in methanol) were prepared. Four types of samples were prepared: cholesterol alone, cholesterol with MCE, cholesterol with DHA, cholesterol with DHA and MCE. Aliquots of cholesterol solution (1 mL) were poured in test tubes. For DHA and MCE containing samples, 1 mL and 0.2 mL of the corresponding stock solution was added, respectively. Solvent was evaporated under a stream of N_2 and uncapped tubes were placed in a dry block (Marconi, Brazil) at 180 °C. After 7 min heating (modelling the medium cooking time for fish), tubes were taken out and introduced into an ice water bath for 4 min and capped. Then, they were kept in the fridge (4 °C) for 72 h or in the freezer (- 30 °C) until analysis. Samples were named as unheated, heated-0 h stored and heated-72 h stored. The experiment was carried out in triplicate.

2.4 Cholesterol and COPs determination

Each sample was dissolved with 1 mL hexane:2-propanol (97:3, v:v), filtered through a 22 µm filter (Millipore, Maryland, MD, USA) and injected into the HPLC system. A Shimadzu liquid chromatograph (Kyoto, Japan) equipped with on-line UV-visible and refractive index detectors was used. The analytical column used was a Nova-Pack CN HP 300 mm x 3.9 mm, 4 µm (Waters, Milford, MA, USA). The mobile-phase was hexane:2-propanol (97:3, v:v) at a flow rate of 1 mL/min and an analysis time of 30 min [27]. 7α-Hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoycholesterol, 5,6α-epoxycholesterol and 7-ketocholesterol were determined. Quantification was done by external standardization. The identification of

COPs was confirmed by HPLC-APCI-MS/MS (HPLC Shimadzu, Kyoto, Japan; MS Bruker Daltonics, model Amazon ETD, Bremen, Germany) using the chromatographic conditions described in detail by Zardetto et al. [28], and the MS conditions previously optimized by Mariutti et al. [27]. Chromatograms of standards separation and MS and MS/MS data and spectra are shown as supplementary material in this manuscript, as well as chromatograms of samples separation (Figures S1-S3 and Table S1).

2.5 DHA determination

Docosahexaenoic acid was converted into its methyl ester according to Joseph & Ackman [29] and analyzed with a gas chromatograph (GC 2010 model, Shimadzu, Kyoto, Japan) equipped with a fused silica CP-SIL 88 capillary column 100 m x 0.25 mm i.d., 0.20 um film thickness (Chrompack, Middelburg, The Netherlands) and flame ionization detector. Chromatographic conditions were described in detail by Sancho et al. [16].

2.6 Statistics

The data obtained were analyzed by means of the software Stata 12 (SataCorp LP, Texas, U.S.A.). For the evaluation of the significant differences among the amounts of cholesterol and COPs of different samples, one factor ANOVA with Bonferroni post hoc multiple comparisons ($p < 0.05$) was applied. For the evaluation of the significant differences between the amounts of cholesterol, COPs and DHA in samples heated and stored for 0 h and 72 h, Student-t test was applied.

3. Results and discussion

3.1 MCE properties

The HPLC-DAD chromatogram processed at 280 nm showed the separation of two phenolic compounds from mana-cubiuextract (Figure 1). The major phenolic compound in the extract was 5-caffeoylquinic acid (5-CQA) (2.48 ± 0.08 mg/g extract), while N^1,N^5 or N^5,N^{10}-bis-(dihydrocaffeoyl) spermidine was found in small amounts (< limit of detection, < 2 μg/mL). Table 1 shows the chromatographic and spectroscopic characteristics of the two identified compounds and the MS and MS/MS spectra are shown in Figure S4. Since a detailed description of the identification of these compounds, in a methanol:water lyophilized mana-cubiu fruit extract was previously reported by Rodrigues et al. [11], these data will not be extensively

discussed. On the other hand, as expected, due to the changes in the polarity of the extraction system and the lower solubility of these compounds in water, the water extract prepared in this work showed lower 5-CQA content than the methanol:water one (4.49 mg/g extract) [11].

Despite its lower phenolic content, the aqueous extract was interesting, since 5-CQA has demonstrated high antioxidant capacity. This compound is usually found in high amounts in coffee, especially green coffee, or coffee extracts, which have been applied both in model and food systems to protect them from oxidation or to increase their antioxidant capacity [30-32]. It is important to highlight that the identified phenolic compounds represent only about 0.25% of the extract composition; therefore, other water soluble compounds such as carbohydrates, proteins, peptides, amino acids, present in the extract probably contributed for the protection against oxidation. On the other hand, this extract was safe, environmentally friendly and potentially applicable in foodstuffs, since it was free from organic solvents. Hence, the aqueous MCE was selected for the experiments carried out in this work.

3.2 Effect of MCE on cholesterol and DHA degradation

The initial amount of cholesterol (before heating) was 1.05 mg (Table 2) in all samples. All samples showed a significant decrease in the cholesterol content after heating (presenting values below 0.82 mg in all cases). As it can be observed in Figure 2, higher amounts of remaining cholesterol were found when MCE was added to the sample (80 %), compared to the remaining amount present when cholesterol was heated alone (55 %). This reduction in cholesterol degradation was attributed to the high content in 5-CQA of MCE and its antioxidant capacity. Similar reductions in cholesterol degradation have also been noticed in other model systems using phenolic compounds such as green tea catechins and quercetin [19, 21].

On the other hand, similar values of remaining cholesterol (around 20%) were found for the two types of samples that included DHA in the mixture, regardless the presence of MCE. As compared to cholesterol alone, the presence of free DHA enhanced cholesterol degradation, and MCE could not counteract this effect. Therefore, MCE seemed to protect cholesterol from oxidation in the absence of DHA, but not in the presence of this fatty acid.

The initial amount of DHA (before heating) was 1.00 mg (Table 2). DHA remaining content after the thermal treatment was also analyzed (Fig 3). Both MCE lacking and containing samples showed a significant decrease in the DHA content after heating (0.11 and 0.36 mg remaining, respectively). Heating of DHA alone (without cholesterol nor MCE) resulted in 11.53 ± 3.05 % of remaining compound, so cholesterol had no effect on DHA thermal degradation given that the presence of cholesterol in the mixture yielded the same remaining amount (11 %). Results showed that DHA content was much higher in the presence of MCE (36 %), compared to the previously mentioned remaining amount found in the absence of the extract (11 %). Similar protective effects of natural extracts against DHA degradation have been also reported in studies dealing with fish meatballs and fish oil [14, 16]. Hence, it could be assumed that MCE antioxidant properties were devoted to protect DHA from degradation, lessening the protective effect towards cholesterol degradation.

3.3 Effect of MCE on COPs formation

After heating, COPs content was much higher in cholesterol-alone sample (227 µg COP / mg cholesterol) than in the presence of MCE (25 µg COP / mg cholesterol), as it can be observed in Table 3. On the other hand, similar values (around 87 µg COP / mg cholesterol) were found for both MCE containing and MCE lacking samples when DHA was present in the medium. So again, as it occurred with cholesterol degradation, MCE seemed to prevent from COPs formation in the absence of DHA, but not in the presence of this compound. COPs formation has been previously reported to be depleted in the presence of phenolic compounds [19, 33].

Nine COPs were analyzed and only five were found in the samples. From these, 7-KC was the main one in most cases, followed by β-EC and 7β-HC (Table 3). A number of studies dealing with cholesterol oxidation in model systems have reported this profile of COPs [19, 33-35]. The dominance of β-isomer was supported by the steric hindrance at C3 position. Interestingly, when comparing cholesterol and cholesterol+MCE samples, whereas a 90 % reduction in 7-KC was noticed; only a 40% reduction was reported in 7α-HC, becoming the main compound. So the antioxidant extract seemed to show differential behavior towards individual COPs formation. In this sense, reaction rate might be slowed down in the presence of MCE,

remaining as 7α-HC for longer time before starting the conversion into 7-KC. Similarly, Kmiecik and co-workers [36] found differences among sterol oxides distribution depending on the antioxidant applied. This selective inhibition towards individual derivatives could be related to the differences in chemical structure, that could hamper certain positions to be attacked and, hence, certain oxidation derivatives to be formed.

3.4 Effect of DHA on cholesterol degradation and COPs formation

Cholesterol degradation was higher when heated within DHA than when heated alone, as it can be observed in figure 2. The presence of a lipid unsaturated surrounding has been reported to protect cholesterol from oxidation [20, 37]. This discordance could be related to the higher ratio cholesterol:lipid matrix used in the current study (1:2) compared to those ones (1:100). Higher amounts of cholesterol could have hampered the physical protection and favoured cholesterol interaction with highly oxidated DHA. This way, Lehtonen and co-workers [26], using cholesteryl esters (stechiometry 1:1) found higher levels of oxidation in cholesteryl linoleate than in free cholesterol (0.17 % and 0.084 %), which was attributed to the linoleate double bonds likelihood to radical formation. Additionally, using free DHA as compared to triglycerides (main constituents of the matrix in [20, 37]) makes also an important difference regarding physical protection, chemical group interaction and viscosity, which are key factors in the process [34].

On the other hand, even though cholesterol degradation was enhanced by DHA, COPs formation was lower than in the absence of DHA, denoting that the routes of cholesterol oxidation were different. Consequently, the oxidation products formed were different, probably oligomers [35, 38, 39] or volatile compounds [35]. It was also possible that reaction rates for COP degradation were higher than for COP formation in the presence of DHA, giving rise to the aforementioned compounds. Previous studies have shown no correlation between the sterols degradation and the oxides formed [24, 40].

3.5 Effect of refrigerated storage

Storage under refrigeration conditions (4 °C, 72 h) modified neither cholesterol levels nor COPs concentration in most cases, except for two samples. Chol+DHA sample slightly decreased its content in COPs, possibly due to degradation of the compounds [35]. DHA levels suffered no changes along the time

either. This behaviour was attributed to the lack of water or any other solvents in the samples, what retarded the oxidation processes.

4. Conclusion

In conclusion, MCE protected against cholesterol degradation and COPs formation when there was no other lipid compound in the system, but not in the presence of DHA. On the other hand, DHA was effectively protected from oxidation by MCE addition. Considering that it implies a solvent-free extraction process, this food-grade mana-cubiu extract could be a potential good ingredient in food products containing highly polyunsaturated lipids to protect them from oxidation and in food products lacking polyunsaturated lipids to protect cholesterol from oxidation.

Acknowledgements

We are grateful to the PIUNA (Plan de Investigación de la Universidad de Navarra) and Ministerio de Economía y Competitividad (AGL2014-52636-P) for their contribution to the financial support of this work. B. Barriuso acknowledges Banco Santander and Asociación de Amigos de la Universidad de Navarra for the grants received. We are grateful to "Red de Excelencia Consolider" PROCARSE (AGL2014-51742-REDC). N. Bragagnolo thanks FAPESP (grant # 2013/06489-1) and CNPq for financial support.

Conflict of interest

Authors have declared no conflict of interest.

References

1. Otaegui-Arrazola A, Menéndez-Carreno M, Ansorena D, Astiasarán I (2010) Oxysterols: A world to explore. Food and Chemical Toxicology 48(12): 3289-3303.

2. Freemantle E, Chen G, Cruceanu C, Mechawar N, Turecki G (2013) Analysis of oxysterols and cholesterol in prefrontal cortex of suicides. International Journal of Neuropsychopharmacology 16(6): 1241-9.

3. Zarrouk A, Vejux A, Mackrill J, O'Callaghan Y, Hammami M, O'Brien N, Lizard G (2014) Involvement of oxysterols in age-related diseases and ageing processes. Ageing Research Reviews 18(0): 148.

4. Meynier A, Andre A, Lherminier J, Grandgirard A, Demaison L (2005. Dietary oxysterols induce in vivo toxicity of coronary endothelial and smooth muscle cells. European Journal of Nutrition 44(7): 393-405.

5. Baumgartner S, Mensink R, Husche C, Lütjohann D, Plat J (2013) Effects of plant sterol- or stanol-enriched margarine on fasting plasma oxyphytosterol concentrations in healthy subjects. Atherosclerosis 227(2): 414-9.

6. Srinivasa K. (2014). Antioxidant potential of spices and their active constituents. Critical Reviews in Food Science and Nutrition 54(3): 352-372.

7. Mariutti LRB, Nogueira GC, Bragagnolo N (2011) Lipid and cholesterol oxidation in chicken meat are inhibited by sage but not by garlic. Journal of Food Science 76(6): C909-C915.

8. Sampaio GR, Saldanha T, Soares RAM, Torres EAFS (2012) Effect of natural antioxidant combinations on lipid oxidation in cooked chicken meat during refrigerated storage. Food Chemistry 135(3): 1383-90.

9. Price A, Díaz P, Bañón S, Garrido MD (2013) Natural extracts versus sodium ascorbate to extend the shelf life of meat-based ready-to-eat meals. Food Science and Technology International 19(5): 427-438.

10. Karwowska M, Dolatowski ZJ (2014) Effect of mustard on lipid oxidation in model pork meat product. European Journal of Lipid Science and Technology 116(3): 311-318.

11. Rodrigues E, Mariutti LRB, Mercadante AZ (2013) Carotenoids and phenolic compounds from Solanum sessiliflorum, an unexploited Amazonian fruit, and their scavenging capacities against reactive oxygen and nitrogen species. Journal of Agricultural and Food Chemistry 61(12): 3022-3029.

12. Mozaffarian D, Wu JHY (2011) Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. Journal of the American College of Cardiology 58(20): 2047-2067.

13. Miyagawa N, Miura K, Okuda N, Kadowaki T, Takashima N, Nagasawa S, Nakamura Y, Matsumura Y, Hozawa A, Fujiyoshi A, Hisamatsu T, Yoshita K, Sekikawa A, Ohkubo T, Abbott RD, Okamura T, Okayama A, Ueshima H (2014) Long-chain n-3 polyunsaturated fatty acids intake and cardiovascular disease mortality risk in Japanese: A 24-year follow-up of NIPPON DATA80. Atherosclerosis 232(2): 384-389.

14. Bhale SD, Xu Z, Prinyawiwatkul W, King JM, Godber JS (2007) Oregano and rosemary extracts inhibit oxidation of long-chain n-3 fatty acids in menhaden oil. Journal of Food Science 72(9): C504-C508.

15. Valencia I, O'Grady MN, Ansorena D, Astiasarán I, Kerry JP (2008) Enhancement of the nutritional status and quality of fresh pork sausages following the addition of linseed oil, fish oil and natural antioxidants. Meat Science 80(4): 1046-1054.

16. Sancho RAS, de Lima FA, Costa GG, Mariutti LRB, Bragagnolo N (2011) Effect of annatto seed and coriander leaves as natural antioxidants in fish meatballs during frozen storage. Journal of Food Science 76(6): C838-C845.

17. Bortolomeazzi R, Cordaro F, Pizzale L, Conte L (2003) Presence of phytosterol oxides in crude vegetable oils and their fate during refining. Journal of Agricultural and Food Chemistry 51(8): 2394-2401.

18. Soupas L, Juntunen L, Lampi AM, Piironen V. (2004). Effects of sterol structure, temperature, and lipid medium on phytosterol oxidation. Journal of Agricultural and Food Chemistry 52(21): 6485-6491.

19. Xu G, Guan L, Sun J, Chen Z (2009) Oxidation of cholesterol and beta-sitosterol and prevention by natural antioxidants. Journal of Agricultural and Food Chemistry 57(19): 9284-9292.

20. Ansorena D, Barriuso B, Cardenia V, Astiasarán I, Lercker G, Rodríguez-Estrada, M (2013) Thermo-oxidation of cholesterol: Effect of the unsaturation degree of the lipid matrix. Food Chemistry 141(3): 2757-64.

21. Chien JT, Hsu DJ, Chen BH (2006) Kinetic model for studying the effect of quercetin on cholesterol oxidation during heating. Journal of Agricultural and Food Chemistry 54(4): 1486-1492.

22. Palozza P, Barone E, Mancuso C, Picci N (2008) The protective role of carotenoids against 7-keto-cholesterol formation in solution. Molecular and Cellular Biochemistry 309(1-2): 61-68.

23. Yen TY, Lu Y, Inbaraj BS, Chen B (2011) Cholesterol oxidation in lard as affected by CLA during heating - a kinetic approach. European Journal of Lipid Science and Technology 113(2): 214-223.

24. Kmiecik D, Korczak J, Rudzinska M, Kobus-Cisowska J, Gramza-Michalowska A, Hes M (2011) beta-Sitosterol and campesterol stabilisation by natural and synthetic antioxidants during heating. Food Chemistry 128(4): 937-942.

25. Xu GH, Sun JL, Liang YT, Yang C, Chen ZY (2011) Interaction of fatty acids with oxidation of cholesterol and beta-sitosterol. Food Chemistry 124(1): 162-170.

26. Lehtonen M, Lampi A, Riuttamaki M, Piironen V (2012) Oxidation reactions of steryl esters in a saturated lipid matrix. Food Chemistry 134(4): 2030-2039.

27. Mariutti LRB, Nogueira GC, Bragagnolo N (2008) Optimization and validation of analytical conditions for cholesterol and cholesterol oxides extraction in chicken meat using response surface methodology. Journal of Agricultural and Food Chemistry 56(9): 2913-2918.

28. Zardetto S, Barbanti D, Rosa MD (2014) Formation of cholesterol oxidation products (COPs) and loss of cholesterol in fresh egg pasta as a function of thermal treatment processing. Food Research International 62(0): 177-182.

29. Joseph JD, Ackman RG (1992) Capillary column gas-chromatographic encapsulated fish oils and fish oil ethyl-ester collaborative study. Journal of AOAC International 75 (3): 488-506.

30. Dupas CJ, Marsset-Baglieri AC, Ordonaud CS, Ducept FMG, Maillard M (2006) Coffee antioxidant properties: Effects of milk addition and processing conditions. Journal of Food Science 71(3): S253-S258.

31. Budryn G, Nebesny E, Rachwal D (2014) Pepsin digestibility and antioxidant activity of egg white protein in model systems with green coffee extract. International Journal of Food Properties 17(7): 1529-1546.

32. Lin C, Toto C, Were L (2015) Antioxidant effectiveness of ground roasted coffee in raw ground top round beef with added sodium chloride. LWT - Food Science and Technolog 60(1): 29-35.

33. Barriuso B, Ansorena D, Calvo MI, Cavero RY, Astiasarán I (2015) Role of Melissa officinalis in cholesterol oxidation: Antioxidant effect in model systems and application in beef patties. Food Research International 69(0): 133-140.

34. Rodriguez-Estrada MT, Garcia-Llatas G, Lagarda MJ (2014) 7-Ketocholesterol as marker of cholesterol oxidation in model and food systems: When and how. Biochemical and biophysical research communications 446(3): 792-797.

288 35. Derewiaka D, Molińska (née Sosińska) E (2015) Cholesterol transformations during heat treatment.
289 Food Chemistry 171(0): 233-240.
290 36. Kmiecik D, Korczak J, Rudzinska M, Gramza-Michalowska A, Hes M (2009) Stabilization of
291 phytosterols in rapeseed oil by natural antioxidants during heating. European Journal of Lipid Science and
292 Technology 111(11): 1124-1132.
293 37. Barriuso B, Poyato C, Astiasarán I, & Ansorena D (2015) Cholesterol and stigmasterol within a
294 sunflower oil matrix: thermal degradation and oxysterol formation. Steroids 99:155-160.
295 38. Lampi A, Kemmo S, Makela A, Heikkinen S, Piironen V (2009) Distribution of monomeric, dimeric and
296 polymeric products of stigmasterol during thermo-oxidation. European Journal of Lipid Science and
297 Technology 111(10): 1027-1034.
298 39. Sosińska E, Przybylski R, Aladedunye F, Hazendonk P (2014) Spectroscopic characterisation of dimeric
299 oxidation products of phytosterols. Food Chemistry 151(0): 404-414.
300 40. Oehrl L, Hansen A, Rohrer C, Fenner G, Boyd L (2001) Oxidation of phytosterols in a test food system.
301 Journal of the American Oil Chemists' Society 78(11): 1073-1078.
302

Table 1. Chromatographic and spectroscopic characteristics of phenolic compounds from mana-cubiu extract.

Compound	tr[a] (min)	λ_{max} (nm)[b]	$[M+H]^+$ (*m/z*)	Fragment ions (*m/z*) MS^2 (+)	$[M-H]^-$ (*m/z*)	fragment ions (*m/z*) MS2 (-)
5-caffeoylquinic acid[d]	18.3	300sh[c], 326	355	163, 145	353	191, 179
N^1,N^5or N^5,N^{10}-bis(dihydrocaffeoyl) spermidine[e]	19.6	280	474	457, 236, 222, 165	472	350, 308, 186

[a] Retention time on the C_{18} column. [b] Solvent: linear gradient of water and acetonitrile both with 0.5% formic acid. [c] sh: shoulder. [d] identified (standard available). [e] tentatively identified. Supplementary Figure S4 presents the MS and MS2 spectra of the identified phenolic compounds.

Table 2. Cholesterol and DHA content (mg) of unheated cholesterol and DHA, and the four heated samples during storage at 4 °C for 0 and 72 h.

	unheated	chol		chol+MCE		chol+DHA		chol+DHA+MCE	
		0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
cholesterol	1.05 aA	0.56 c	0.54 C ^{ns}	0.82 b	0.81 B ^{ns}	0.22 d	0.22 D ^{ns}	0.25 d	0.29 D *
DHA	1.00 aA	-	-	-	-	0.11 c	0.07 C *	0.36 b	0.41 B ^{ns}

Different lower case letters denote statistical differences among the unheated sample and the heated samples stored for 0 h.
Different capital letters denote statistical differences among the unheated sample and the heated samples stored for 72 h.
ns: non-significantly different content between heated samples stored for 0 and 72 h within each type of sample.
*: significantly different content between heated samples stored for 0 and 72 h within each type of sample.

Table 3. Cholesterol oxidation products (µg/mg cholesterol) content of the unheated sample and the four heated samples during storage at 4 °C for 0 and 72 h.

	unheated	chol		chol+MCE		chol+DHA		chol+DHA+MCE	
		0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
7α-HC	nd	28.90 c	27.78 C ^{ns}	17.07 b	9.55 B *	6.47 a	0.18 A *	10.42 ab	0.18 A *
7β-HC	nd	48.25 c	47.17 C ^{ns}	1.84 a	4.74 A *	13.01 b	11.06 B ^{ns}	13.25 b	15.04 B ^{ns}
β-EC	nd	61.06 c	63.12 C ^{ns}	1.21 a	1.11 A ^{ns}	20.78 b	15.29 B *	20.11 b	18.49 B ^{ns}
α-EC	nd	29.22 c	31.81 C ^{ns}	0.65 a	0.59 A ^{ns}	15.33 b	10.78 B *	12.58 b	10.88 B ^{ns}
7-KC	nq	59.65 c	68.37 C ^{ns}	3.26 a	7.97 A *	31.10 b	25.71 B *	30.78 b	30.64 B ^{ns}
Total COPs	-	227.07 c	238.24 C ^{ns}	24.03 a	23.96 A ^{ns}	86.69 b	63.02 B *	87.14 b	75.22 B ^{ns}

Different lower case letters denote statistical differences among heated samples stored for 0 h. Different capital letters denote statistical differences among heated samples stored for 72h.
nd: not detected (detection limit: 7α-HC = 0.98 µg/mg, 7β-HC = 0.46 µg/mg, β-EC = 4.99 µg/mg, α-EC = 3.67 µg/mg)
nq: not quantitated (quantification limit: 7-KC = 1.01 µg/mg)
ns: non-significantly different content between heated samples stored for 0 and 72 h within each type of sample.
*: significantly different content between heated samples stored for 0 and 72 h within each type of sample.

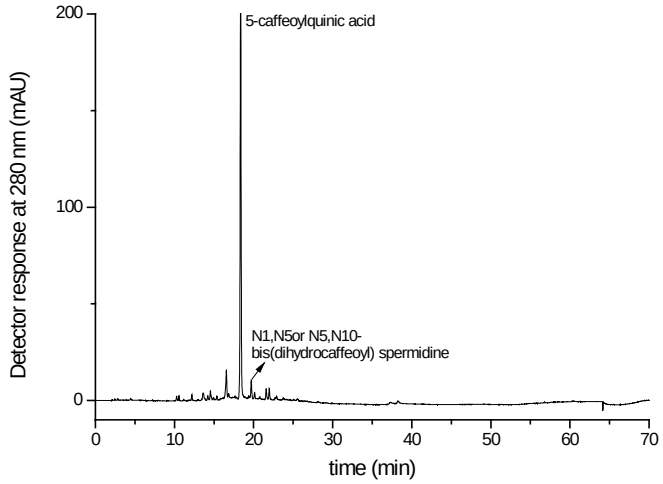


Figure 1. Chromatogram obtained by HPLC-DAD of the phenolic compounds from the aqueous mana-cubiu extract.

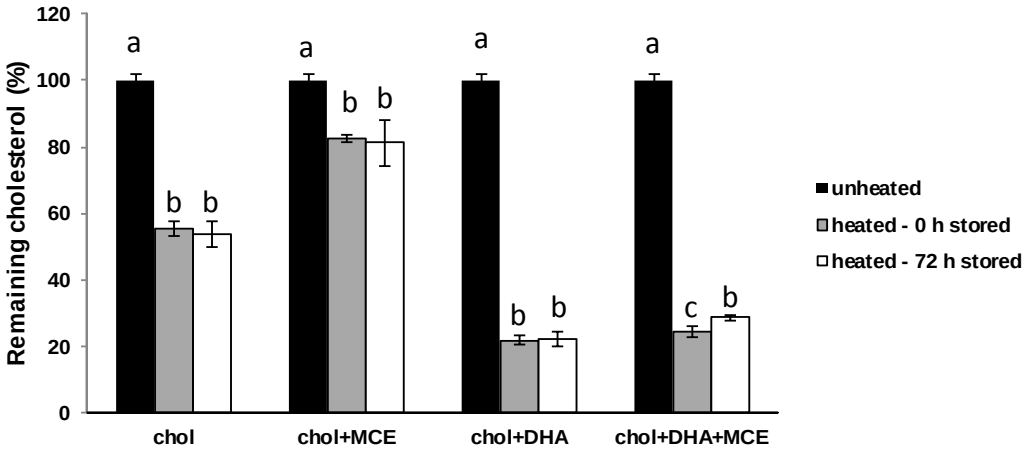


Figure 2. Remaining percentage of cholesterol of the unheated sample and the four heated samples after 0 and 72 h storage. Different letters for each sample denote statistical differences among the unheated, the 0 h stored and the 72 h stored samples.

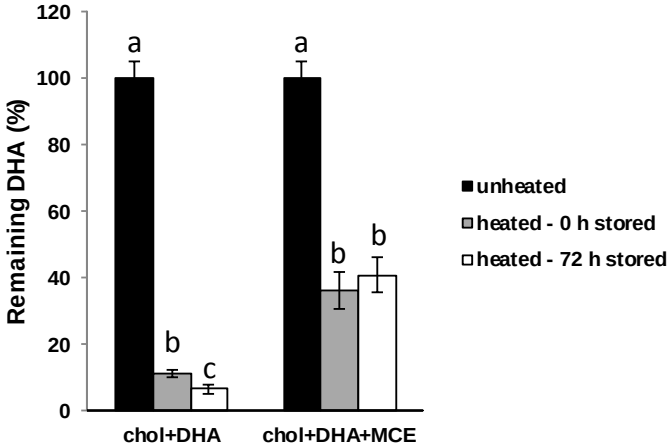


Figure 3. Remaining percentage of DHA of the unheated sample and the two heated samples after 0 and 72 h storage. Different letters for each sample denote statistical differences among the unheated, the 0 h stored and the 72 h stored samples.

Figure captions

Fig. 1 Chromatogram obtained by HPLC-DAD of the phenolic compounds from the aqueous mana-cubiu extract

Fig. 2 Remaining percentage of cholesterol of the unheated sample and the four heated samples after 0 and 72 h storage. Different letters for each sample denote statistical differences among the unheated, the 0 h stored and the 72 h stored samples

Fig. 3 Remaining percentage of DHA of the unheated sample and the two heated samples after 0 and 72 h storage. Different letters for each sample denote statistical differences among the unheated, the 0 h stored and the 72 h stored samples

Fig. S1 Extracted ion chromatograms at *m/z* 385 and *m/z* 401, obtained by HPLC-MS/MS, of cholesterol oxide standards. Peak characterization is given in Table S1.

Fig. S2 Extracted ion chromatograms at *m/z* 385 and *m/z* 401, obtained by HPLC-MS/MS, of cholesterol oxides in model system. Peak characterization is given in Table S1.

Fig S3 Mass spectra of cholesterol oxide standards.

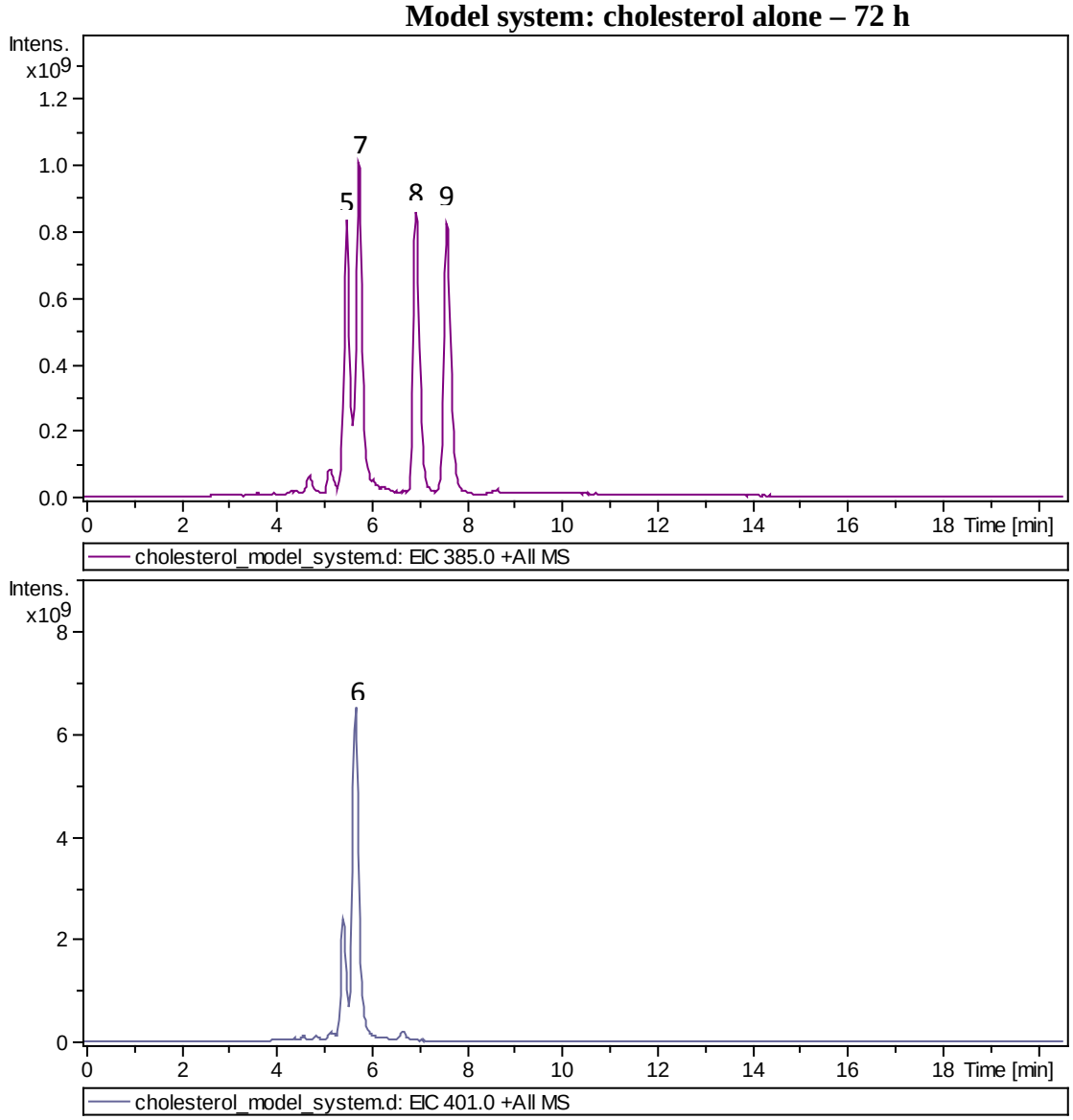
Fig. S4 Mass spectra of the phenolic compounds in mana-cubiu water extract.

Suplementary Table S1. Chromatographic and mass spectrometry characteristics of cholesterol oxides obtained by HPLC-MS/MS.

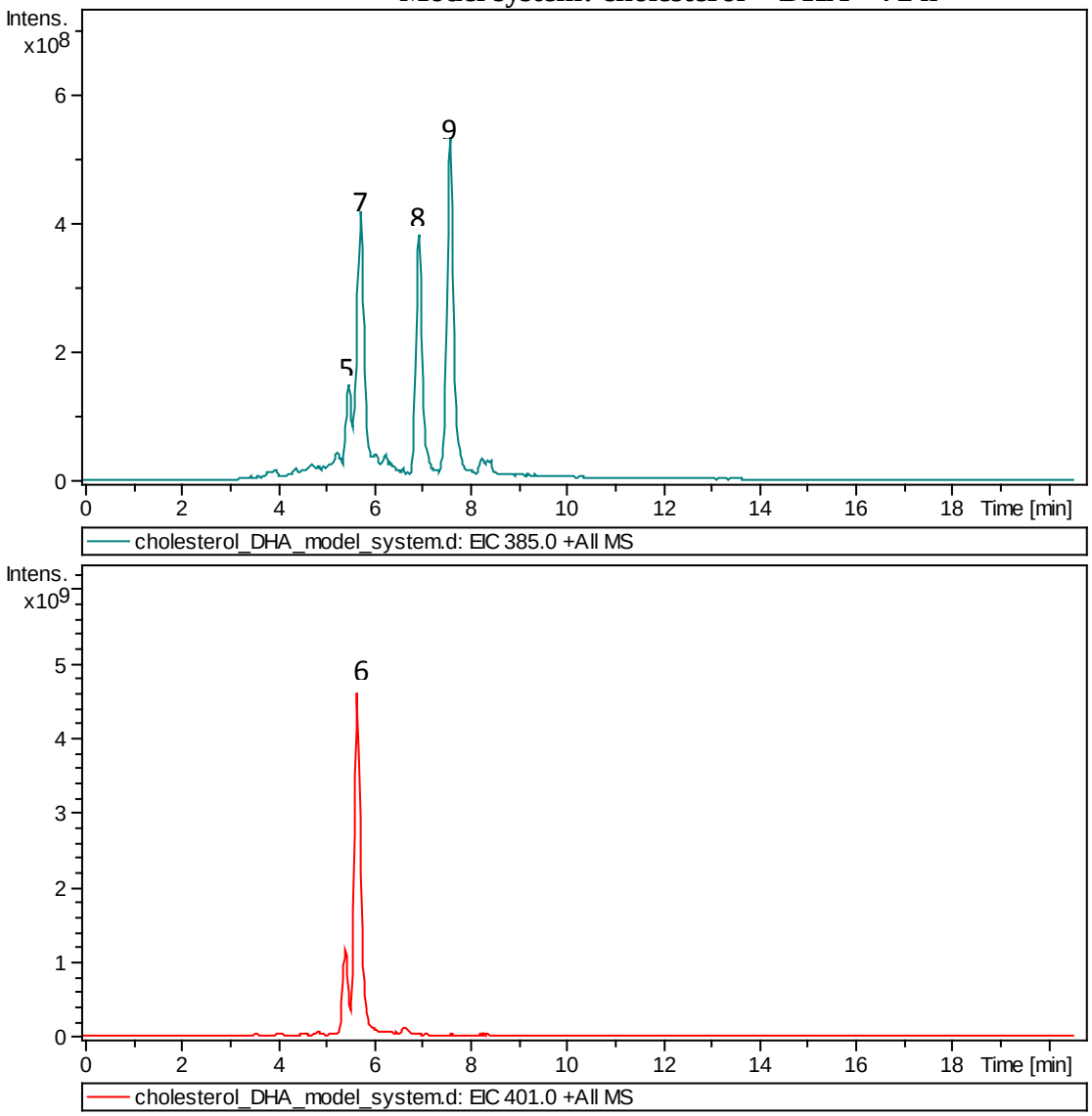
Cholesterol oxide	t_r (min)	$[M+H]^+$ *(m/z)*	Fragment ions *(m/z)*
22R-hydroxycholesterol	3.7	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
22S-hydroxycholesterol	4.2	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
20α-hydroxycholesterol	4.4	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
25-hydroxycholesterol	4.7	403	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$
7α-hydroxycholesterol	5.5	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
7-ketocholesterol	5.7	401	383 $[M+H-18]^+$, 365 $[M+H-18-18]^+$
7β-hydroxycholesterol	5.8	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
5,6β-epoxycholesterol	7.0	403	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$
5,6α-epoxycholesterol	7.6	403	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$

nd: Not detected. * In source fragmentation.

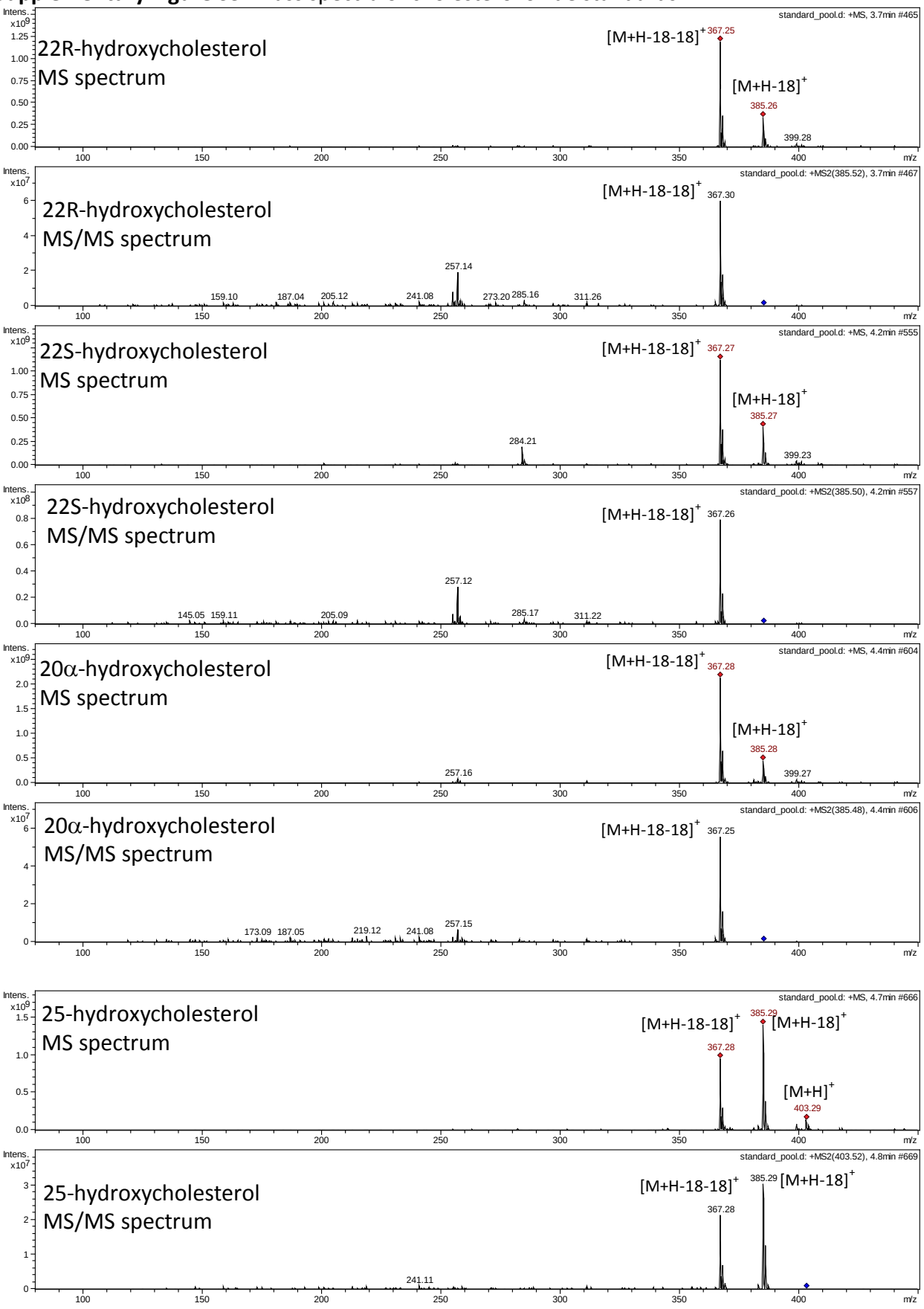
Supplementary Figure S2. Extracted ion chromatograms at *m/z* 385 and *m/z* 401, obtained by HPLC-MS/MS, of cholesterol oxides in model system. Peak characterization is given in Table S1.

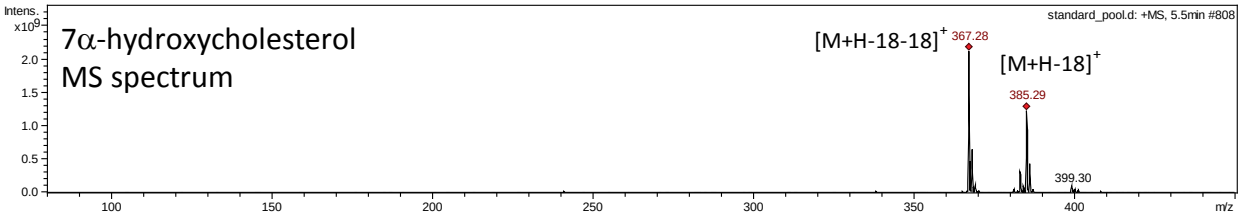


Model system: cholesterol + DHA – 72 h

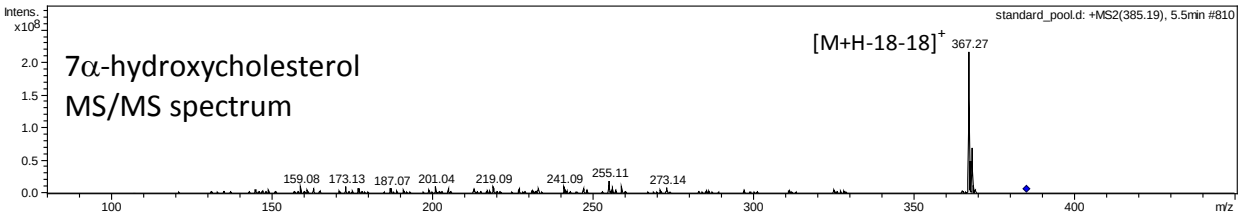


Supplementary Figure S3. Mass spectra of cholesterol oxide standards.

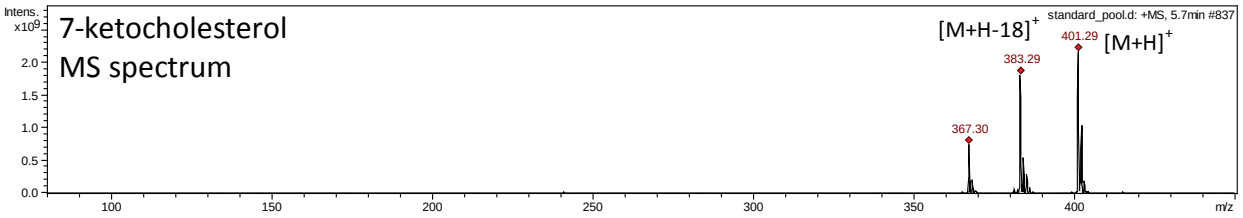




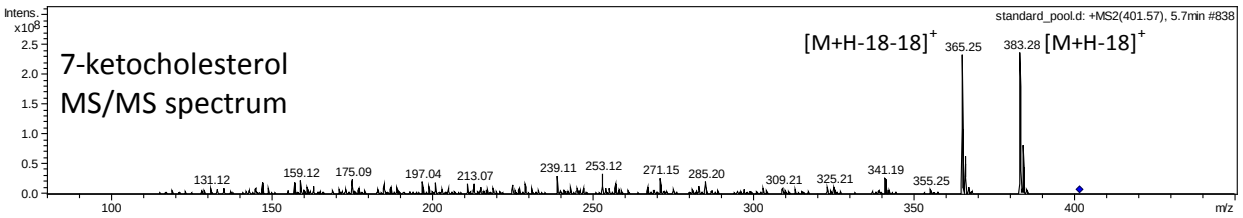
386



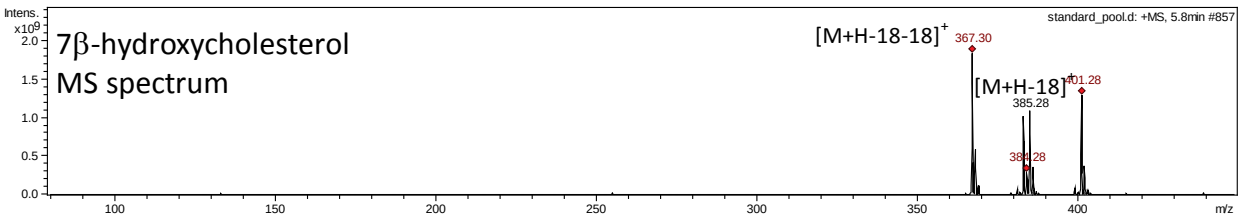
387



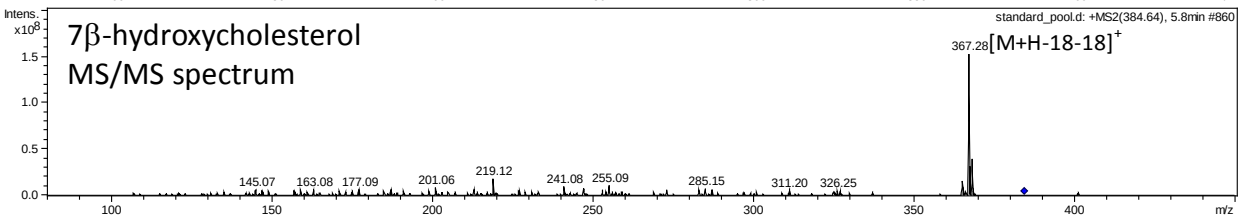
388



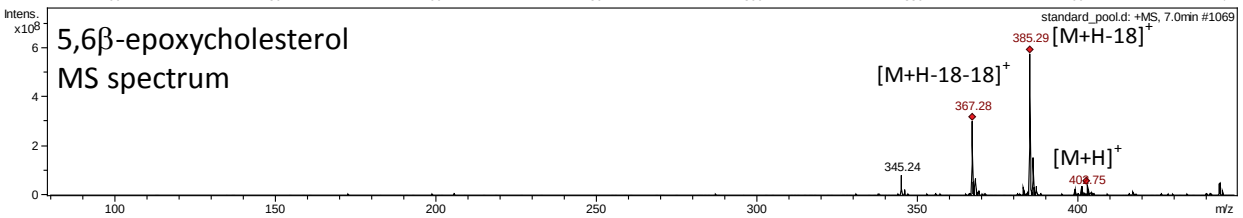
389



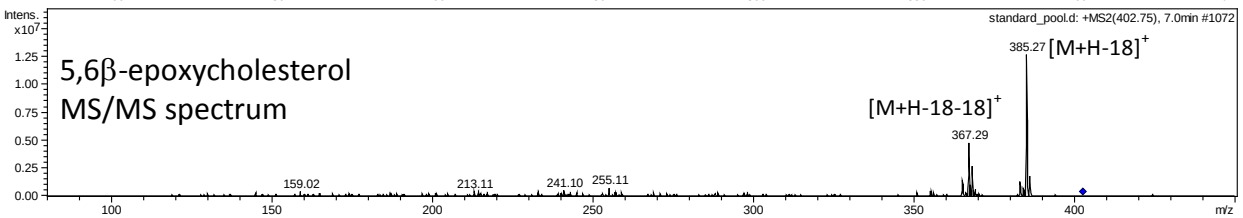
390



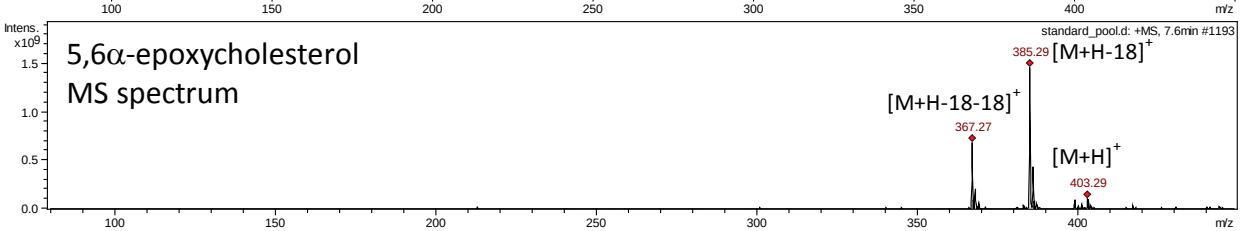
391



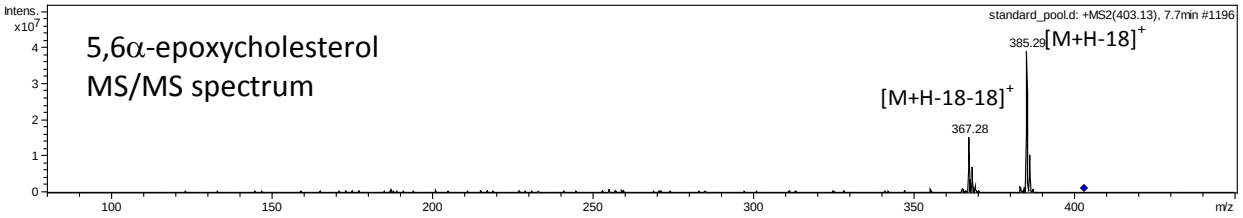
392



393



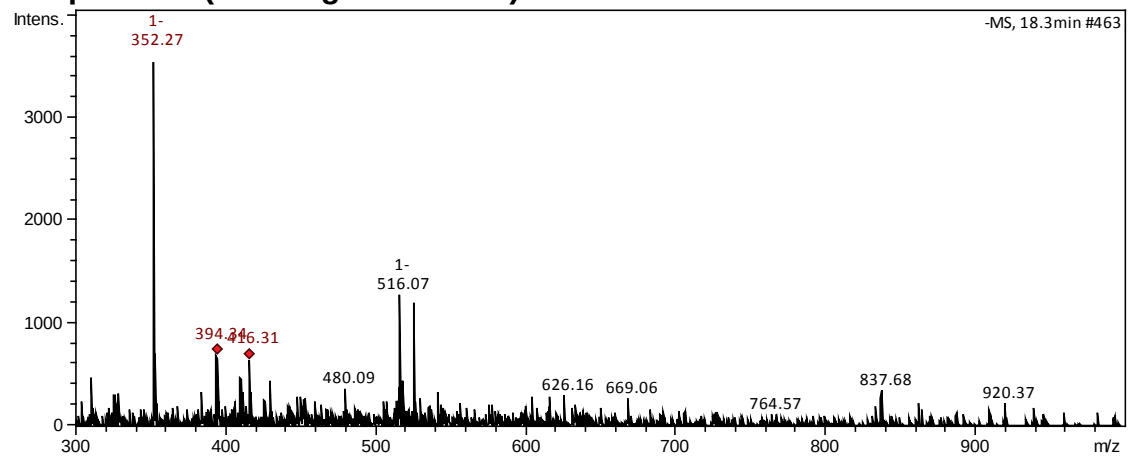
394


Intens.
x10^7
4
3
2
1
0
100
150
200
250
300
350
400
m/z
standard_pool.d: +MS2(403.13), 7.7min #1196
5,6α-epoxycholesterol
MS/MS spectrum
385.29
[M+H-18]+
[M+H-18-18]+
367.28

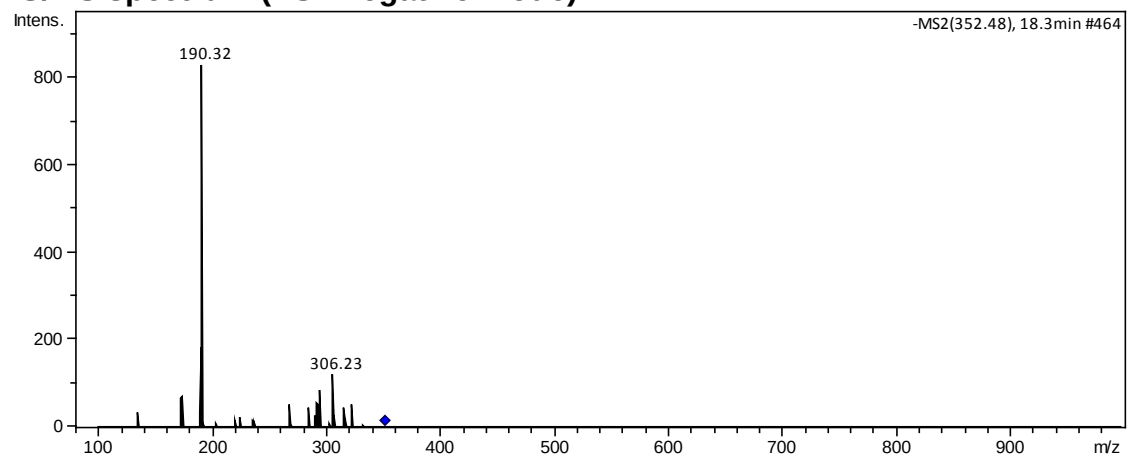
Supplementary Figure S4. Mass spectra of the phenolic compounds in mana-cubiu water extract.

5-CQA

MS spectrum (ESI- negative mode)

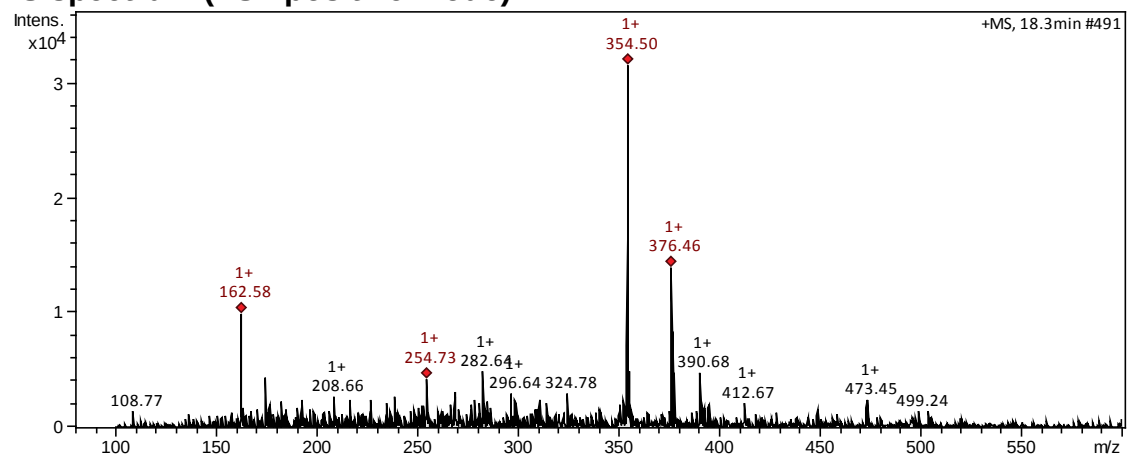


MS/MS spectrum (ESI- negative mode)

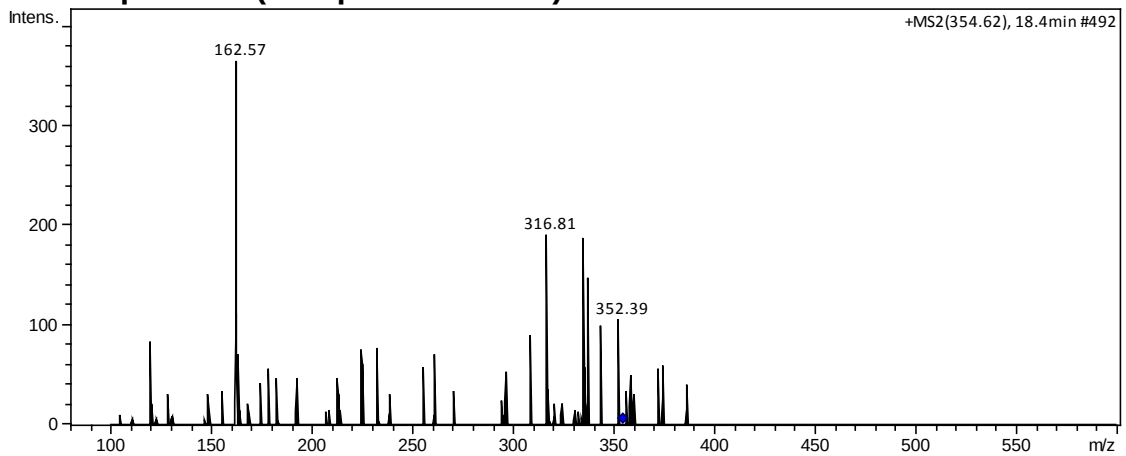


5-CQA

MS spectrum (ESI- positive mode)

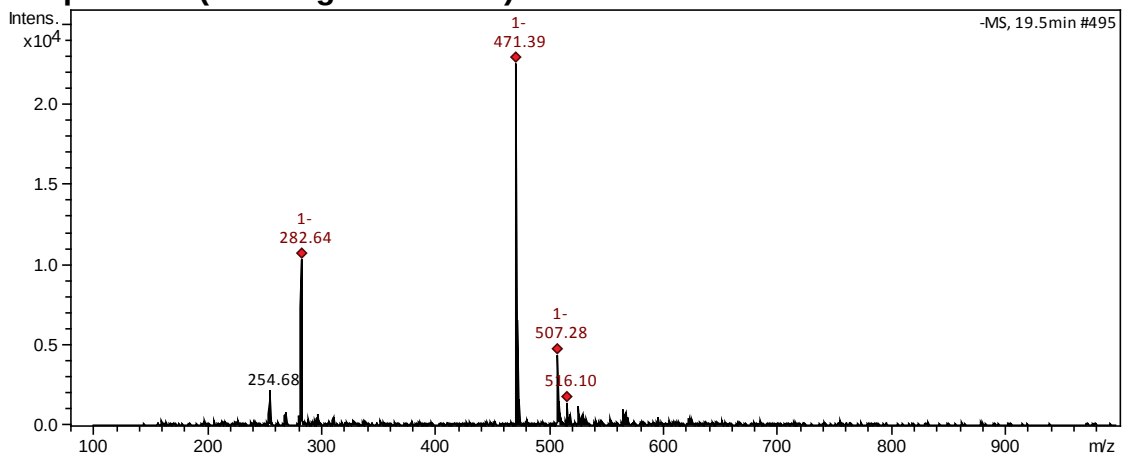


MS/MS spectrum (ESI- positive mode)

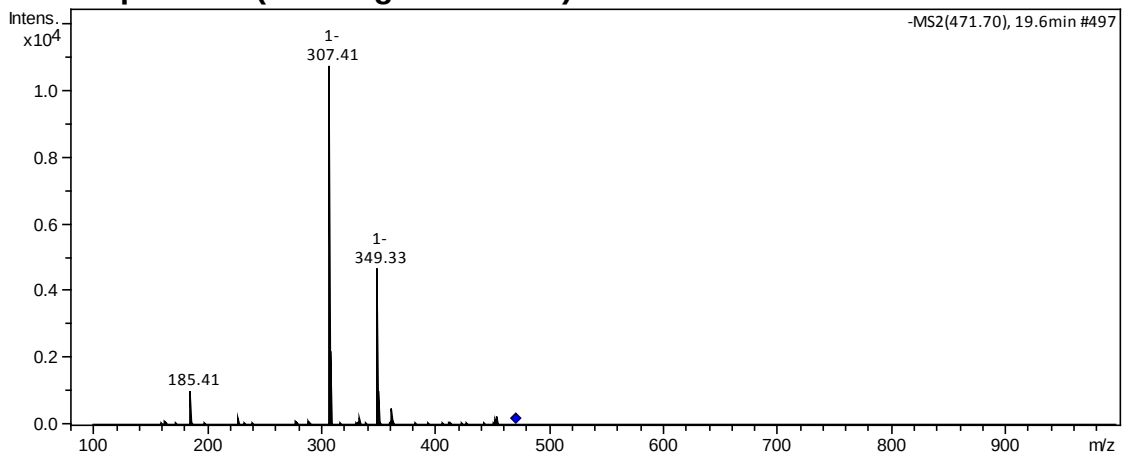


N_1 ,N_5 or N_5 ,N_{10} -bis-(dihydrocaffeoyl)spermidine

MS spectrum (ESI- negative mode)

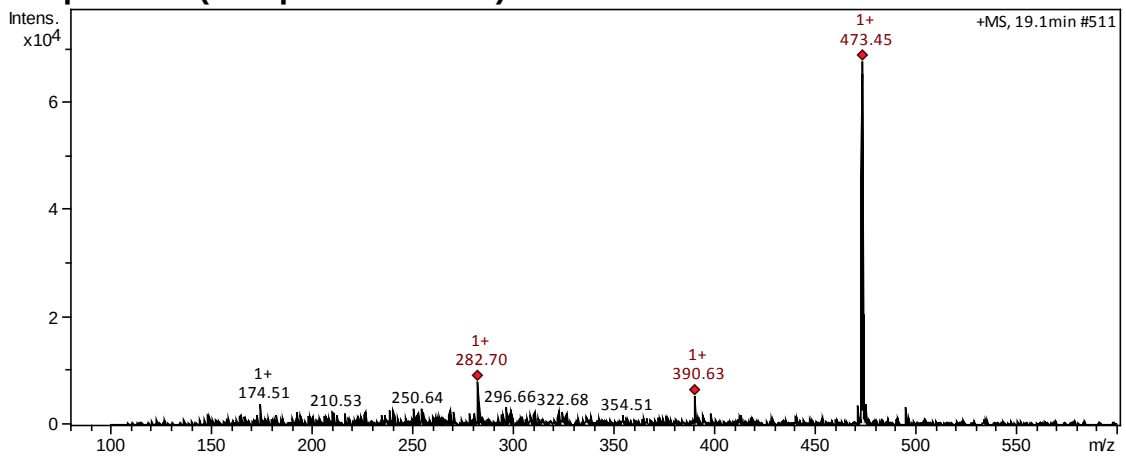


MS/MS spectrum (ESI- negative mode)



N_1 ,N_5 or N_5 ,N_{10} -bis-(dihydrocaffeoyl)spermidine

MS spectrum (ESI- positive mode)



MS/MS spectrum (ESI- positive mode)