

# Analysis on antibacterial activities and volatile compounds of Maillard reaction products derived from squid skin

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**Abstract:** Maillard reaction products (MRPs) could offer flavor and aroma for food, and also render functional characteristics. In this work, three squid skin Maillard reaction products with glucose (GSS), fructose (FSS) and lactose (LSS) were prepared, whose volatile compounds were analyzed by gas chromatography-mass spectrometer. And the antibacterial activities of three Maillard reaction products against *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* and *Vibrio harveyi* were investigated. The results showed that the volatile compounds of squid skin MRPs mainly contained alcohol, acid, phenol, ester, aldehyde, alkane, alkene, amide and ester, among which alcohol was the predominant component in the GSS and LSS, accounting for 53.87±6.02 % and 83.67±3.64%, respectively, however, acid was predominant component in the FSS, accounting for 54.19±3.38 %. Surprisingly, 2,6-di-tert-butyl-4-methylphenol (BHT), as a synthetic antioxidant, was firstly identified in these three MRPs, which directly indicated squid skin MRPs have antioxidant activities. It is demonstrated that the antibacterial abilities of LSS and GSS were superior to that of FSS, and *Escherichia coli* was the most sensitive to the three MRPs among the three tested bacteria. This study demonstrates squid skin MRPs possess antioxidant and antibacterial abilities, which shows promising and far-ranging prospect in the fields of food additives.

## 1 Introduction

Maillard reaction is a chemical reaction which occurs between free or protein-bound amino acids and reducing sugars [1]. This reaction generates a variety of early, intermediate and advanced compounds [2], which is of high significance for the food industry, playing a crucial role during thermal processing of foods [3-4]. Furthermore, it has been proven with the ability to modify the sensory characteristics [5-6], contributing to the aroma, flavor and color [7]. Maillard reaction products (MRPs), have attracted a lot of attention recently, being considered as functional food ingredients due to their potential pro-healthy properties [3], including antioxidative, antimicrobial, antihypertensive, anticarcinogenic and antimutagenic properties [2].

Importantly, there are some evidences that antimicrobial properties of MRPs could also contribute to extending shelf-life of food products by inhibiting growth of spoilage-causing organisms [8-9]. Thus, in recent years, the antimicrobial effect of MRPs has attracted considerable attention. It has been proven that MRPs can inhibit the growth of different strains of bacteria like *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Aeromonas hydrophila*, and *Helicobacter pylori* [10-15]. This important functional property may be derived

simultaneously, and naturally, during food processing and storage, which can substitute chemical bacteriostatic agents. At present, the antimicrobial properties of MRPs generated in commercial food products such as coffee, beer and biscuit have also been widely investigated [16-17].

The squid, one of cephalopods species, is widely distributed in tropical and subtropical waters [1]. It is one of the commercially important aquatic products in China. At present, approximately 800 thousand tons squid are processed per year, mainly in Zhejiang, Shandong and Fujian, approximately 20 % by-products of squid are skin, being considered as waste materials, were discarded in the factories, which is not only harmful to the environment, but also a waste of resource. Squid skin is rich in protein, including 75% collagen, and also rich in vitamins and minerals, mainly used to extract collagen [18] and prepare gelatin [19], polysaccharides [20] and polypeptides, such as angiotensin-I-converting enzyme (ACE) inhibitory peptide activity [21]. Therefore, the utilization of squid skin for more value-added materials is an economical and environmental advantage. However, to our best knowledge, there has been not any reports of comparatively analyzing the antimicrobial activities of squid skin MRPs with different reducing sugars.

Therefore, the aim of the present study was to prepare squid skin MRPs with three reducing sugars (glucose,

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fructose and lactose), analyze the volatile compounds released from MRPs by GC–MS, and investigate their antimicrobial activities with three bacteria (*Escherichia coli*, methicillin-resistant *Staphylococcus aureus* and *Vibrio harveyi*).

## 2 Materials and methods

### 2.1 Materials and chemicals

The fresh squid (*Doryteuthis singhalensis*) skin was collected from an aquatic processing enterprise in Zhoushan, Zhejiang, China. Alkaline protease was purchased from Pangbo Biological Engineering Co., Ltd. (Shandong, China). Glucose, fructose, lactose, *Escherichia coli* (*E. coli*), methicillin-resistant *Staphylococcus aureus* (MRSA), *Vibrio harveyi* (*V. harveyi*), phenanthroline, ferrous sulfate (FeSO<sub>4</sub>), hydrogen peroxide, pyrogallol, potassium ferricyanide, trichloroacetic acid (TCA), and ether were obtained from Sinopharm Chemical Reagent Co., Ltd. (Hangzhou, China). All chemicals and reagents used were of analytical grade.

### 2.2 Preparation of squid skin enzymatic hydrolysate

The fresh squid skin was stored at -20 °C until use. The frozen squid skin was thawed with flowing water, cleaned thoroughly in iced water. 100 g squid skin and 1000 mL aqueous solution were homogenized by a tissue crusher and rinsed with deionized water until pH was neutral. Then, 500mL squid skin homogenate and 10g alkaline protease were hydrolyzed at 52 °C for 2 h to obtain the squid skin hydrolysate, deactivated in water bath at 85 °C for 10 min, rapidly cooled in ice water, and then centrifuged at 6000 r/min for 5 min. The prepared squid skin enzymatic hydrolysate was stored at 4 °C.

### 2.3 Preparation of MRPs

50 mL squid skin hydrolysate were mixed with 1 g glucose, fructose and lactose respectively in the conical flasks, adjusted the pH to 7 with 0.1 mol/L HCL or 0.1 mol/L NaOH, and heated in water bath at 100 °C for 4h. When the reaction was completed, the conical flask was immediately placed in ice water and cooled for 30 min to inhibit the reaction from proceeding. The prepared MRPs samples were obtained and denoted as GSS (glucose MRPs sample), FSS (fructose MRPs sample) and LSS (lactose MRPs sample) respectively.

### 2.4 Volatile compounds analysis of MRPs by GC-MS

10 g MRPs was added to 50 mL ether to extract volatile compounds for 3 h, and a pale-yellow extract was obtained. The ether extract was volatilized naturally in the centrifuge tube until the volume was 1 mL, and the concentrated sample was stored in air-tight sample vessel.

The volatile compounds of extracting oily liquid were measured by GC-MS (2010, Shimadzu, Japan).

GC-MS was equipped with a DB-5MS capillary column (30 m × 0.25 μm × 25 mm). The initial temperature was maintained at 50 °C for 1 min, and then raised to 250 °C at a rate of 10 °C/min. All samples were injected in split mode at 250 °C. The mass spectrometer was operated in EI mode (positive ion, 70 eV), and the quadrupole was 200 °C. Mass spectra were acquired in full scan mode with repetitive scanning from 20 m/z to 500 m/z for 1 s.

### 2.5 Bacterial strain and culture conditions

*E. coli* (DSM 498, ATCC 23716) were cultivated aerobically under agitation in nutrient broth for 24 h at 37 °C. MRSA were obtained from laboratory collection, which were grown on MHB agar plates supplemented with 1.4% agar and incubated at 37 °C for 24 h under aerobic conditions. *V. harveyi* (BB170) were grown at 37 °C for 24 h in autoinducer bioassay (AB) medium. Cell counting was performed by plating 1 mL of an adequate serial decimal dilution in plate count agar. The plates were incubated for 24 h at 37 °C and the cells were counted manually [11]. The three kinds of bacteria were separated with culture medium respectively, and then diluted with PBS.

### 2.6 Antimicrobial activity of MRPs

The MRPs solutions were diluted to 1 %, 2 %, 4 %, 8 % and 16 % with distilled water, respectively. 100 μL 10<sup>8</sup> CFU/mL bacteria suspension were added into 100 mL MRPs solutions with different concentrations. Take 250 μL mixed solution as the sample group and 250 μL bacteria suspension without MRPs as the control group, and transfer them into the wells of 96-well microplate. The assay was incubated aerobically for 24 h at 37 °C during agitation. The absorption at 630 nm of each well was recorded by using UV–Vis spectrophotometer (UV 2600, Shimadzu, Japan). The experiments were performed in three independent repetitions. The bacteriostatic rate of MRPs was calculated by the following formula.

$$R(\%) = \frac{C - S}{C}$$

Where R is bacteriostatic rate (%), C is OD<sub>630nm</sub> value of the control group and S is OD<sub>630nm</sub> value of the sample group.

## 3 Results and discussion

### 3.1 Volatile components analysis of MRPs

The volatile components of GSS, FSS and LSS are shown in Table 1. It can be seen that a total of 22, 23 and 16 chemical constituents are identified from the volatile components of GSS, FSS and LSS, containing alcohol, acid, phenol, ester, aldehyde, alkane, alkene and amide. As illustrated in Figure 1, alcohol is the predominant component in the GSS and LSS samples, accounting for

53.87±6.02 % and 83.67±3.64%, respectively, among which 20-Dihydro-11-deoxycortisol, also called cholesterol, has the highest content in GSS and LSS, accounting for 52.89±5.85 and 73.61±3.20 %, respectively. And acid is predominant composition in the FSS, accounting for 54.19±3.38 %, mainly including palmitic acid, docosahexaenoic acid, stearic acid, oleic acid, etc. As we know, glucose and lactose belong to aldose, fructose is the ketose, and lactose is a dimer made of glucose and galactose. Thus, GSS and LSS have similar volatile compositions.

2,6-di-tert-butyl-4-methylphenol, tetra decanoic acid, tetra decanal, palmitic acid, cis-11-eicosenoic acid and (Z)-docos-13-enamide are all identified in these three MRPs. Interestingly, 2,6-di-tert-butyl-4-methylphenol

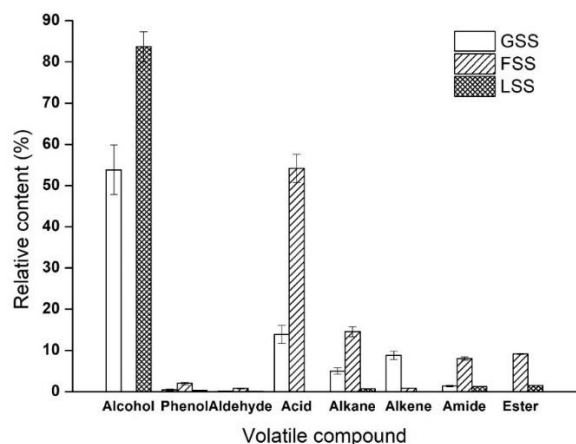
(BHT), also named butylated hydroxytoluene, is a synthetic antioxidant. Thus, GSS, FSS and LSS can exhibit antioxidant activities. And tetra decanoic acid and tetra decanal are common food additives, which can endow food with unique flavor.

Synthetic antioxidants are mostly used in the food industry owing to their high performance, low cost, stability and wide availability. BHT is one of the most widely used synthetic antioxidants [22]. However, in our study, BHT was first discovered in the MRPs of squid skin, which was also found in the mangrove leaves [23], rendering its antioxidant capacity of MRPs of squid skin. Thus, MRPs of squid skin as natural food additives could exhibit extensive prospects of its application in food, feedstuff and medicine industry.

**Table 1.** The volatile compounds of GSS, FSS and LSS by GC-MS

Number	Compound name	GSS content (%)	FSS content (%)	LSS content (%)
1	1-dodecanol	0.13±0.04	-	-
2	2,4-di-tert-butylphenol	0.03±0.04	-	-
3	2,6-di-tert-butyl-4-methylphenol	0.40±0.17	2.00±0.27	0.34±0.03
4	cyclotetradecan	0.10±0.03	-	-
5	tetra decanoic acid	0.19±0.06	0.71±0.03	0.10±0.02
6	tetra decanal	0.22±0.04	0.80±0.10	0.14±0.04
7	palmitoleic acid	0.13±0.04	-	-
8	palmitic acid	4.18±1.03	19.39±1.96	3.05±0.05
9	heptadecanoic acid	0.12±0.11	-	-
10	20-dihydro-11-deoxycortisol	52.89±5.85	-	73.61±3.20
11	stearic acid	7.06±0.61	7.77±0.29	-
12	heptadecane	1.57±0.03	-	-
13	icosa-5,8,11,14-tetraenoic acid	0.47±0.09	-	-
14	Methyl-eicosa-5,8,11,14,17-pentenoate	2.17±0.37	-	-
15	cis-11-eicosenoic acid	1.53±0.23	4.88±0.11	0.52±0.04
16	9-octadecenamide	0.68±0.13	-	-
17	3β-hydroxy-5,24-cholestadiene	8.78±1.01	-	-
18	Docosa-4,7,10,13,16,19-hexaensaeure-methylester	3.14±0.62	-	-
19	n-hexakosan	0.22±0.22	-	-
20	(Z)-docos-13-enamide	0.71±0.04	6.87±0.31	0.75±0.04
21	5,8,11,14,17-eicosapentaenoic acid	0.18±0.19	-	-
22	cholest-3,5-dien	0.84±0.13	-	-
23	1-decene	-	0.83±0.02	-
24	1,2-cyclopropylundecane	-	0.32±0.13	-
25	margaric acid	-	0.68±0.07	0.10±0.02
26	oleic acid	-	4.28±0.33	-
27	hexadecane	-	1.60±0.05	-
28	docosane	-	0.74±0.65	-
29	icosane	-	6.31±0.27	0.68±0.09
30	arachidonic acid	-	1.12±0.07	-
31	5,8,11,14,17-eicosapentaenoic acid methyl ester	-	9.16±0.11	1.51±0.02
32	oleamide	-	1.18±0.11	0.52±0.01
33	tetracosane	-	2.49±0.09	-
34	docosahexaenoic acid	-	14.29±0.33	1.55±0.03
35	hexacosane	-	2.41±0.30	-
36	octadecane	-	0.70±0.61	-

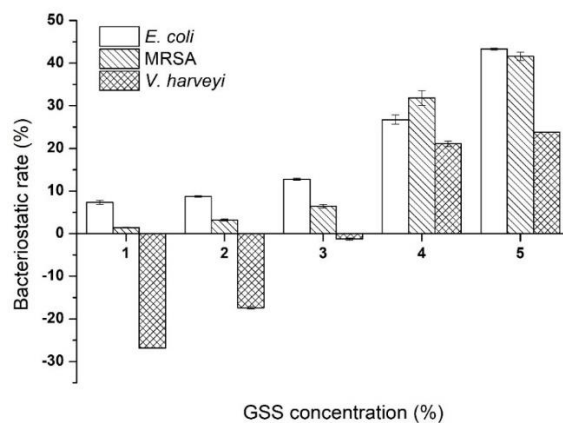
37	2-(2,6-dimethyl-4-(3-oxo-3-(4-(2,2,2-trifluoroethylthio) phenyl) propyl) phenoxy)-2-methylpropanoic acid	-	1.62±0.06	-
38	(8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R)-6-methylheptan-2-yl]-2,7,8,9,11,12,14,15,16,17-decahydro-1H-cyclopenta phenanthrene	-	1.29±0.19	1.02±0.03
39	desmosterol	-	-	10.5±0.46
40	pentosane	-	-	0.23±0.04
41	1,2-octadecylene oxide	-	-	0.06±0.02



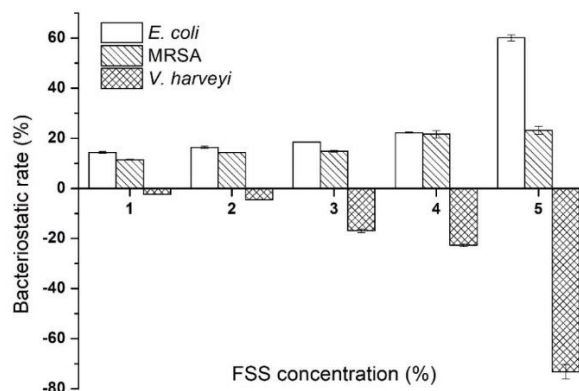
**Figure 1.** Relative contents of the main volatile compositions in GSS, FSS and LSS

### 3.2 Antibacterial abilities of GSS, FSS and LSS MRPs

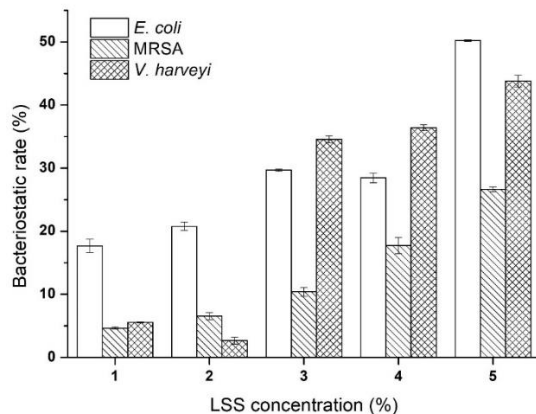
As shown in Figure 2, Figure 3 and Figure 4, these three MRPs samples demonstrate the antibacterial abilities against *E. coli*, MRSA and *V. harveyi* at different degree. And *E. coli* is the most sensitive to the three MRPs among the three tested bacteria. The results indicate that the antibacterial effects of LSS are stronger than those of GSS and FSS against the three tested bacteria. Overall, the antibacterial effect of squid skin MRPs samples against the three tested bacteria are improved with the increase of MRPs concentration, except FSS against *V. harveyi*, whose growth inhibition has not been observed. The inhibitory effects of MRPs against bacteria appear to depend mostly on three primary factors, namely, the species of bacteria, the physical-chemical nature of MRPs and the external factors that influence the kinetics of Maillard reaction (e.g. temperature and duration, pH and water content) [24-25].



**Figure 2.** Bacteriostatic effect of GSS against *Escherichia coli* (*E. coli*), methicillin-resistant *Staphylococcus aureus* (MRSA), *Vibrio harveyi* (*V. harveyi*).



**Figure 3.** Bacteriostatic effect of FSS against *Escherichia coli* (*E. coli*), methicillin-resistant *Staphylococcus aureus* (MRSA), *Vibrio harveyi* (*V. harveyi*).



**Figure 4.** Bacteriostatic effect of LSS against *Escherichia coli* (*E. coli*), methicillin-resistant *Staphylococcus aureus* (MRSA), *Vibrio harveyi* (*V. harveyi*).

In this study, the species of bacteria and the physical – chemical nature of MRPs are primary factors. The species of bacteria are important factors to determine the antibacterial effect of MRP, e.g. Gram-positive bacteria and Gram negative bacterial have significantly different sensitive to the same MRPs. Daglia and co-workers had reported that Gram-positive bacteria were relatively more sensitive to coffee MRP derived inhibition, compared to Gram-negative bacteria [26]. However, in this study, a greater antibacterial effect was observed against Gram negative bacterial (*E. coli*) in squid MRPs. Thus, it will be a tremendous challenge to a define a specific antimicrobial activity relationship with one or more bacteria owing to the complexity of MRP composition. The physical – chemical nature of MRPs is a critical factor to affect the antibacterial ability of MRP. MRP is a complicated mixture system, among which melanoidin is the final products of the Maillard reaction, whose chemical structure has not been completely understood, although it is generally agreed that they comprise anionic, brown-colored, high molecular weight, nitrogen containing compounds [27]. It has been verified that some MRPs particularly melanoidins have dramatically antibacterial activities [10, 28-29]. And it is found that the inhibitory effect of MRPs was dependent on the degree of browning [9], whose is direct correlation with reducing power of sugar participating in Maillard reaction [30]. As is known, glucose and lactose as aldoses, whose reducing power is stronger than fructose as a ketose. Thus, in this study, the antibacterial abilities of LSS and GSS are superior to that of FSS.

## 4 Conclusion

In summary, this study provides a comprehensive comparative analysis of volatile compounds of squid skin MRPs with glucose, fructose and lactose by using the GC-MS. It is demonstrated that alcohol is the predominant component in the GSS and LSS, among which cholesterol has the highest content, and acid is predominant composition in the FSS. Interestingly, 2,6-di-tert-butyl-4-methylphenol, as a synthetic antioxidant, is identified in these three MRPs, which indicates squid skin MRPs could exhibit antioxidant activity. In addition, the antibacterial

activities of the three MRPs against *E. coli*, MRSA and *V. harveyi* are also investigated, among which *E. coli* is the most sensitive to the three MRPs. This work proves that squid skin MRPs display antioxidant and antibacterial effect, which would be a promising functional ingredient to be used in the food additives.

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## References

1. A. Veeruraj, L. Liu, J. Zheng, J. Wu, M. Arumugam, Mater. Sci. Eng. C **95**, 2942 (2019)
2. F.L. Gu, J.M. Kim, S. Abbas, X.M. Zhang, S.Q. Xia, Z.X. Chen, Food Chem. **120**, 505-511 (2010)
3. E. Langner, W. Rzeski, Int. J. Food Prop. **17**, 344-353 (2014)
4. H.F. Erbersdobler, V. Somoza, Mol. Nutr. Food Res. **51**, 423-430 (2007)
5. C.K. Wei, K. Thakur, D.H. Liu, J.G. Zhang, Z.J. Wei, Food Chem. **263**, 186-193 (2018)
6. K. Chen, X. Yang, Z. Huang, S. Jia, Y. Zhang, J. Shi, H. Hong, L. Feng, Y. Luo, Food Chem. **295**, 569-578 (2019)
7. L.N. Vhangani, J. Van Wyk, Food Chem. **208**, 301-308 (2016)
8. N. Banerjee, R. Bhatnagar, L. Viswanathan, Appl. Microbiol. Biot. **11**, 226-228 (1981)
9. R. Lanciotti, M. Anese, M. Sinigaglia, C. Severini, R. Massini, LWT - Food Sci. Technol. **32**, 223-230 (1999)
10. H. Einarsson, B.G. Snygg, C. Eriksson, J. Agric. Food Chem. **31**, 1043-1047 (1983)
11. K. Hiramoto, T. Kida, K. Kikugawa, Biol. Pharm. Bull. **25**, 1467-1471 (2002)
12. A. Tauer, S. Elss, M. Frischmann, P. Tellez, M. Pischetsrieder, J. Agric. Food Chem. **52**, 2042-2046 (2004)
13. G.P. Rizzi, Food Rev. Int. **13**, 1-28 (1997)
14. V.T. Trang, H. Takeuchi, H. Kudo, A. Aoki, S. Katsuno, T.T. Shimamura, T. Sugiura, H. Ukeda, J. Agric. Food Chem. **57**, 11343-11348 (2009)
15. J.M. Ames, A. Wynne, A. Hofmann, S. Plos, G G.R. ibson, Br. J. Nutr. **82**, 489-495 (1999)
16. J.A. Rurián-Henares, F.J. Morales, J. Agric. Food Chem. **56**, 2357-2362 (2008)
17. J.A. Rurián-Henares, F.J. Morales, Food Chem. **111**, 1069-1074 (2008)

18. D.A. Cuevas-Acuña, R.M. Robles-Sanchez, W. Torres-Arreola, E. Marquez-Rios, J.M. Ezquerra-Brauer, *CyTA–J. Food* **14**, 193-199 (2015)
19. B.E. Abdelmalek, J. Gómez-Estaca, A. Sila, O. Martinez-Alvarez, M.C. Gómez-Guillén, S. M. Chaabouni-Ellouz, A. Ayadi, A. Bougatef, *LWT–Food Sci. Technol.* **65**, 924-931 (2016)
20. N.K Karamanos, A.J. Aletras, C.A. Antonopoulos, T. Tsegenidis, C.P. Tsiganos, D.H. Vynios, B. B. A. **966**, 36-43 (1988)
21. A. Alemán, M.C. Gómez-Guillén, P. Montero, *Food Res. Int.* **54**, 790-795 (2013)
22. Y. Zhang, Y. Shen, Y. Zhu, Z. Xu, *LWT–Food Sci. Technol.* **63**, 569-574 (2015)
23. Z. Wang, D.L. Yu, C.F. Zheng, Y.N. Wang, L. Cai, J. Guo, W.D. Song, L.L. Ji, *J. Mar. Sci. Eng.* **7**, 292 (2019)
24. S.I. Martins, W.M. Jongen, M.A. Boekel, *Trends Food Sci. Tech.* **11**, 364-373 (2000)
25. F. Jousse, T. Jongen, W. Agterof, S. Russell, P. Braat, *J. Food Sci.* **67**, 2534-2542 (2002)
26. M. Daglia, R. Tarsi, A. Papetti, P. Grisoli, C. Dacarro, C. Pruzzo, G. Gazzani, *J. Agric. Food Chem.* **50**, 1225-1229 (2002)
27. F.J. Morales, *Food Chem.* **76**, 363-369 (2002)
28. C. Hauser, U. Müller, T. Sauer, K. Augner, M. Pischetsrieder, *Food Chem.* **145**, 608-613 (2014)
29. J.A. Rufián-Henares, F.J. Morales, *Food Res. Int.* **40**, 995-1002 (2007)
30. H. Wang, H. Qian, W. Yao, *Food Chem.* **128**, 573-584 (2011)