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Polyphenol Content in Argentinean Commercial Extra Virgin Olive Oil

*Gabriela Castelli, Ismael D. Bianco, and Roxana Kiyomi Mizutamari**

Olive oil polyphenols are unique among all polyphenols, the only ones approved to claim health benefits. Since 2012, the European Commission has authorized the use of the claim "olive oil contributes to the protection of blood lipids from oxidative stress" only for those oils containing at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil. Therefore, in order to examine the health attributes of olive oil from Argentinean producers in relation to that claimed effect, the content of hydroxytyrosol and tyrosol and their secoiridoid derivatives in commercial extra virgin olive oil (EVOO) is evaluated. For this purpose, a simple and reliable methodology for the quantification of tyrosol and hydroxytyrosol by HPLC after hydrolysis of EVOO polar fraction, which leads to the release of hydroxytyrosol and tyrosol moieties from their conjugated forms has been implemented and validated. Two of the eleven analyzed EVOOs reach the polyphenol concentration required to use the above-mentioned health claim if the mass differences between hydroxytyrosol, tyrosol, and their conjugated forms are considered in the calculation of their content. This study provides a current landscape of the polyphenol content in EVOOs produced in Argentina that can be helpful for the producers and also for the consumers, in the light of the health claim approved by the European Commission.

***Practical Applications*: Applying a new validated simple and reliable methodology, only 2 of 11 analyzed commercial Argentinean EVOOs present the hydroxytyrosol, tyrosol, and their conjugated form concentrations, according to the requirement to claim such a health effect, such as prevention of LDL oxidation. Thus, Argentinean olive oil producers can be encouraged to upgrade their production regarding to polyphenol contents.**

1. Introduction

Polyphenols are secondary metabolites of plants that we incorporate through our diet from fruits, vegetables, cereals, cocoa, olive oil, and beverages such as coffee, wine, and tea. They have received great attention because of their antioxidant capacity and the correlation between their consumption and the prevention of certain diseases including cardiovascular diseases, type 2 diabetes, cancers, and the promotion of other health benefits.[1–3]

In light of the continuous growth incidence of these "preventable diseases," as warned by the World Health Organization based on their strong association with lifestyle, mainly nutrition patterns and low physical activity,[4] consumption of foods containing polyphenols could help to ameliorate this situation.[5]

Nevertheless, any beneficial-effect health claims on foodstuffs are regulated by the corresponding country authorities, who approve the health claims on food labeling based on rigorous scientific substantiation.

So far, the only food that has been authorized to claim a health benefit based on its polyphenol content is olive oil, and in no other place outside of the European Union (EU).[6] There does exist a health claim on specific cocoa products, related to their flavonol content, which is also authorized by the EU, but its use is restricted to a single Zurich-based producer.[7]

Since 2012, the European Commission Regulation (EU) 432/2012 has authorized the following health claim on olive oil: "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress."[6] Prevention of LDL oxidation is thought to provide a beneficial physiological effect, since oxidized LDL are pro oxidant causing tissue injury and promoting the development of atherosclerotic lesions.[8–11]

It was established that the claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil. Additionally, information regarding that "the beneficial effect is obtained with a daily intake of 20 g of olive oil" shall be given to the consumer.[6]

The polyphenols involved in this health claim belong to secoiridoid group that is the main group among the complex polyphenol fraction of olive oil, accounting for 80% or more of

G. Castelli, Prof. I. D. Bianco, Prof. R. K. Mizutamari
Centro de Excelencia en Productos y Procesos de Córdoba (CEPROCOR)
Santa María de Punilla, Córdoba X5164, Argentina
E-mail: kiyomi.mizutamari@gmail.com
Prof. I. D. Bianco
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
Santa María de Punilla, Argentina
Prof. I. D. Bianco, Prof. R. K. Mizutamari
Universidad Nacional de La Rioja
La Rioja, Argentina
Prof. R. K. Mizutamari
Facultad de Odontología
Universidad Nacional de Córdoba
Córdoba, Argentina

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the total content.[12] This group is constituted by oleuropein and ligstroside derivatives, containing in their molecules hydroxytyrosol (Htyr, 3,4-DHPEA) and tyrosol (Tyr, *p*-HPEA) moieties, respectively. Among them, the most common determined compounds are the dialdehydic forms of decarboxymethyl elenolic acid linked to Htyr (3,4-DHPEA-EDA or oleacein) or Tyr (*p*-HPEA-EDA or oleocanthal), and oleuropein and ligstroside aglycons (3,4-DHPEA-EA and *p*-HPEA-EA, respectively).[13]

Also verbascoside, a caffeic ester present in the fruit, contains Htyr; but has not been reported to be present in the olive oil.[14]

It has been asserted that, among the different olive oil categories, extra virgin olive oil (EVOO) as defined by Codex Alimentarius has the highest polyphenol content. However, the polyphenol composition, quality, and quantity in olive oil depends on several factors, including cultivar, climate, soil composition, agricultural practices (fertilization and irrigation), and oil production process.[15–17]

Most of the available data about polyphenol composition in olive oil comes from the Mediterranean basin, which is the world's major production area. In addition, only few studies have addressed the polyphenol content in olive oil available on market.[18,19]

During recent years, a growing number of small olive oil mills have been installed in Argentina. Therefore, in an attempt to evaluate the health attributes of Argentinean EVOO related to the above-mentioned claimed effect, we analyzed the content of Htyr and its derivatives in commercial EVOOs available in local markets. However, it is well known that the determination of these polyphenols is a complex task; because of their poor resolution by HPLC as well as GC,[20] and the absence of commercial standards for most of them. Recently, the artificial formation of isomers during HPLC analysis has been demonstrated, which leads to broadened peaks in chromatograms.[21]

The use of polyphenol hydrolysis in such a way that allows the release of Htyr and Tyr from the molecules containing them has been applied in different studies for accurately determining the polyphenols involved in the claim.[18,22–25] Therefore, we implemented and validated a simple and reliable methodology for the quantification of Tyr and Htyr by HPLC after hydrolysis of olive oil polar fraction that could be used to support the health claim on olive oil polyphenols content based on the Commission Regulation (EU).

2. Experimental Section

2.1. Reagents

Hydroxytyrosol and tyrosol were purchased from Extrasynthese (Genay, France). Analytical grade sulfuric acid and HPLC grade methanol (MeOH), acetonitrile and phosphoric acid were supplied by Sintorgan (Argentina).

Ultrapure water was obtained through a Milli-Q system (Millipore, Argentina).

2.2. Standard Solutions

Stock standard solutions of 3 mg L^{-1} of Tyr and Htyr were prepared by dissolving these substances in a mixture of methanol/water (80/20, v/v).

Calibration samples were made in the range of 5–30 mg L^{-1} for Htyr and Tyr.

2.3. Olive Oil Samples

EVOOs were purchased from local markets in Córdoba Province (Argentina). The EVOOs used in our study do not provide information regarding variety of olive.

2.4. Polyphenol Extraction

Polyphenols were extracted from EVOO following the International Olive Council method (IOC).[20] Briefly, 2.0 g of the EVOO sample were weighed, in a 10 mL screw cap tube, 1 mL of MeOH/H_2O (80/20, v/v) or of standard solution of Htyr or Tyr for recovery assays was added and shaken for 30 s, after 5 mL of MeOH/H_2O (80/20, v/v) was added and shaken again for 1 min. The extraction was performed in an ultrasonic bath for 15 min at RT. Then the tube was centrifuged at 5000 rpm for 25 min.

2.5. Acidic Hydrolysis

Acidic hydrolysis was carried out according to Mastralexi et al.[23] that is based on Mulinacci et al.[25] Briefly, an aliquot of EVOO polar extract was mixed with the same volume of a 1 M H_2SO_4 solution. The mixture was incubated in a water bath at 80 °C for the time indicated. The hydrolysates were filtered through a 0.45 μm pore size PVDF membrane (Durapore) to be injected directly into the chromatograph.

2.6. HPLC Analysis for Polyphenol Determination

The HPLC analysis was carried out according to the IOC method.[20] A chromatograph (Waters 2690) equipped with a diode array detector (Waters 996) was used, set at 280 nm. Briefly, for separation a C18 column, 250 × 4.6 mm (5 μm) (Phenomenex Luna) was used, with a ternary linear elution gradient as mobile phase. The gradient was initiated with 0.2% H_3PO_4 (v/v) 96%, MeOH 2%, and acetonitrile 2%, and finalized 0.2% H_3PO_4 (v/v) 0%, MeOH 50%, and acetonitrile 50%. A mobile phase flow of 1 mL min^{-1} was used.

3. Results and Discussion

3.1. Validation of the Quantitative Analytical Method for Htyr and its Derivatives in Olive Oil

Firstly, the hydrolysis of EVOO polar extract through H_2SO_4 at 80 °C was evaluated at different times; following the % Tyr and % Htyr as compared to non-hydrolyzed polar extract (**Table 1**).

After 2 h the amount of Htyr and Tyr was almost duplicated, which did not significantly increase after a further 2 h; indicating that the complete hydrolysis of complex forms of both compounds had already been reached.

Table 1. Acidic hydrolysis of polyphenol extract of an EVOO.

	Extra virgin olive oil extract		Standards	
Time (h)	%Htyt (RSD)	%Tyr (RSD)	%Htyr (RSD)	%Tyr (RSD)
0	100 (9)	100 (3)	100 (3)	100 (1)
1	160 (5)	188 (4)	nd	nd
2	191 (5)	223 (2)	108 (3)	102 (2)
4	198 (4)	233 (2)	111 (2)	106 (1)

Htyr and Tyr were quantified after hydrolysis by HPLC at 280 nm. The obtained quantities are expressed as % mean ± RSD (relative standard deviation) respect to $t = 0$ (100%) from $n = 3$. nd, not determined.

Under the assayed hydrolysis conditions, Htyr and Tyr standard solutions, showed to be stable.

As shown in **Figure 1**, after hydrolysis of EVOO polar extract, the corresponding peaks to Htyr and Tyr increased. They were the main polyphenols detected, even before hydrolysis (0.34–0.49 mg Htyr $20\,g^{-1}$ oil and 0.29–0.57 mg Htyr $20\,g^{-1}$ oil from two different olive oils). It has been highlighted that the concentration of both compounds may increase during storage because of hydrolysis of the complex forms (linked moieties).[26]

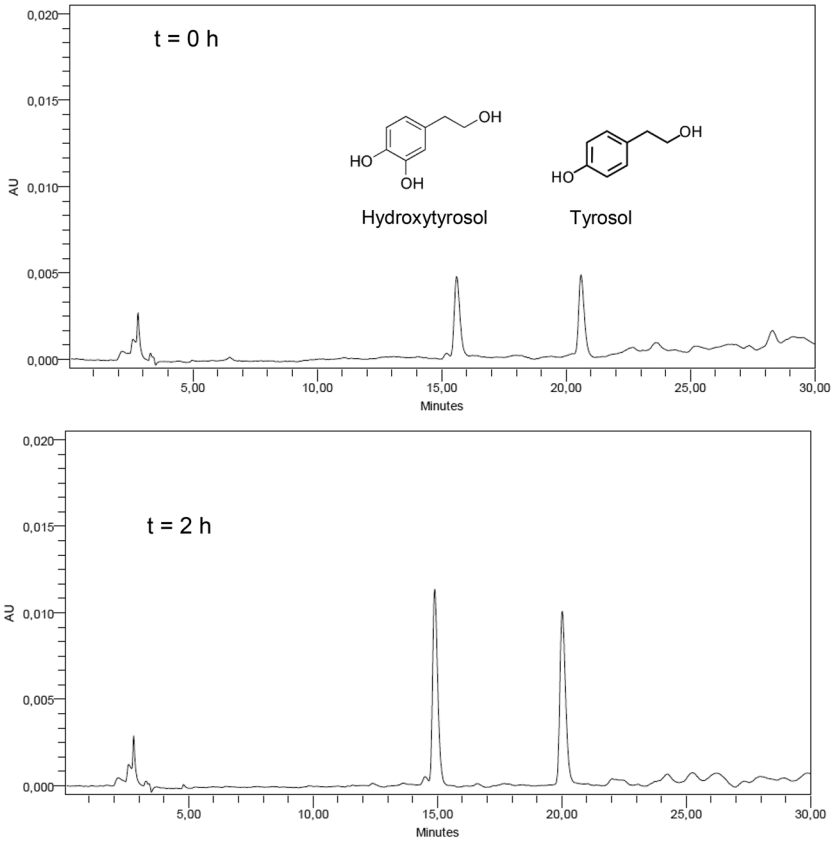

Figure 1. Acidic hydrolysis of EVOO extract. Chromatograms of a representative sample of EVOO polar extract, before and after hydrolysis for 2 h, recorded at 280 nm. Samples were injected directly into the chromatograph.

The Htyr and Tyr content were analyzed, before and after hydrolysis, in two EVOO samples with low and high content. It was observed that the initial content of Htyr and Tyr increased around 2.2 times after hydrolysis of oleuropein and ligstroside compounds, respectively, in both samples; indicating that both forms are present at an approximately equal amount in mole terms.

To quantify Htyr and Tyr in the hydrolyzed polar extract of EVOO, a HPLC method that consisted in the simultaneous determination of Htyr and Tyr using their respective calibration curves was validated.

For this purpose, sensitivity, precision, and recovery parameters were estimated following ICH (International Conference on Harmonization) recommendations.

3.1.1. Sensitivity

The sensitivity of the method was evaluated as limit of detection (LOD) and limit of quantification (LOQ) base on signal-to-noise approach. It was considered a signal-to-noise ratio equal to 3 and 10 for estimating the LOD and LOQ, respectively.

The calculated values shown in **Table 2** allow the quantification of amounts as low as $0.16\,\mu g\,mL^{-1}$ Htyr and $0.3\,\mu g\,mL^{-1}$ Tyr, which are lower than those reported elsewhere.[22,23] Therefore, it is very suitable for the intended application.

3.1.2. Precision

It was evaluated by determining simultaneously the Htyr and Tyr concentrations in EVOO following the standardized method. The corresponding assays were carried out with replicated samples ($n = 3$) and on different days ($n = 3$) for interday and intraday precision analysis, respectively.

The calculated values as relative standard deviation (RSD) as shown in Table 2 are an acceptable level.

3.1.3. Recovery

For recovery evaluation, EVOO samples were spiked before polyphenol extraction with Htyr or Tyr dissolved in MeOH/H_2O (80/20, v/v) at levels that are present in the two analyzed olive oils with low and high concentration of both compounds (Table 2).

The recovery was calculated as the ratio of the mean concentration of each compound determined in the spiked EVOO samples against those theoretically calculated from three independent measurements.

As shown in Table 2, Htyr and Tyr were almost fully recovered from two samples.

Table 2. Method validation for determination of Htyr and its derivatives in EVOO.

Parameter	Htyr	Tyr
Sensitivity		
LOD	0.05 μg mL^{-1}	0.1 μg mL^{-1}
LOQ	0.16 μg mL^{-1}	LOQ 0.3 μg mL^{-1}
Precision		
Intraday	RSD % 1.3–3.9	RSD % 3.1–3.6
Interday	RSD % 2.8	RSD % 4.8
Recovery		
Low	% Recov (RSD) 100 (7)	% Recov (RSD) 97 (5)
High	% Recov (RSD) 100 (4)	% Recov (RSD) 99 (6)

3.2. Evaluation of EVOO Produced in Argentina

Following the standardized and validated method, EVOOs available in the local market of Córdoba, Argentina were evaluated with regard to Htyr and its derivatives content (**Table 3**).

Htyr and Tyr were present at different ratios ranging from 0, i.e., non-Htyr content, to 1.5, and only two of all the analyzed samples contained more Htyr than Tyr. However, none of them presented a total amount of Htyr and its derivatives (Htyr + Tyr) $\geq$ 5 mg 20 g^{-1} oil.

Overall, our data show lower concentrations of polyphenol in analyzed olive oils determined as total content of Htyr and Tyr (0.156–3.09 mg 20 g^{-1} oil) after acidic hydrolysis, compared to those from the Mediterranean basin (3.6–7.6 mg 20 g^{-1} oil,[18] 1.76–3.73 mg 20 g^{-1} oil,[23] 1.3–8.4 mg 20 g^{-1} oil).[24]

Since complex forms, i.e., secoiridoid derivatives, have a higher molecular weight than their respective free forms, the mass differences between both forms should be considered, when they are quantified after the linked moieties breakage.[22,23]

Based on the mean molecular weight of the most known secoiridoids, a factor of 2.2 and 2.5 was proposed to correct the calculated amount of Htyr and Tyr, respectively.[23] Taking this into consideration, the content of Htyr and its derivatives was over 5 mg 20 g^{-1} in two of the analyzed EVOOs.

It should be worth mentioning that for the protection of blood lipids from oxidative stress, it is irrelevant whether they are consumed in olive oil as free or conjugated form, since both forms are absorbed and the complex compounds undergo fast hydrolysis.[27] Furthermore, Htyr and Tyr are the molecular species incorporated into LDL preventing its oxidation.[27]

Recently, the polyphenol content of olive oil produced in a specific region of Argentina (Mendoza) from olive with the same ripening index, and applying the same extraction method, was characterized by Liquid Chromatography-Mass Spectrometry. The data show that 8/25 of the oils analyzed contain over 5 mg of Htyr and its derivatives per 20 g, determined by the sum of Hty, Tyr, and secoiridoids. Furthermore, oils from the same cultivar exhibited great variation, with a factor of 2 being the greatest difference between them.[28]

The composition of olive oil produced in Argentina under controlled conditions (ad hoc for the study) showed that an increase of total polyphenol content due to the decrease of olive fruit ripening, was mainly related to an increase of secoiridoid content.[29] Therefore, adjusting the olive ripening stage could improve the healthy attribute of Argentinean oil.

4. Conclusion

The aim of this study was to evaluate olive oils produced in Argentina acquired in the marketplace with regard to their health promoting attributes associated with their polyphenol composition in terms of Htyr and its derivatives. The content of polyphenols in commercial samples of olive oils from Argentina has previously been described, but they were quantified in terms of total content by the Folin-Ciocalteu method.[30] Our results show that 2 of the 11 samples of EVOO analyzed could claim health benefit.

The method based on HPLC, implemented to quantify the Htyr and its derivatives, after hydrolysis of EVOO polar extract is simple and reliable. It also allows the estimation of oleuropein and ligstroside derivatives, from the difference between the

Table 3. Htyr and Tyr contents in commercial EVOO (mg 20 g^{-1} oil).

Sample	Tyr (mg 20 g^{-1} oil ± SD)	Htyr (mg 20 g^{-1} oil ± SD)	Tyr + Htyr (mg 20 g^{-1} oil ± SD)	Tyr + Htyr Fc[a)] (mg 20 g^{-1} oil ± SD)
L	0.156 ± 0.001	ND	0.156 ± 0.001	0.391 ± 0.003
G	0.305 ± 0.005	0.062 ± 0.001	0.367 ± 0.006	0.90 ± 0.01
H	0.208 ± 0.003	0.319 ± 0.001	0.527 ± 0.004	1.22 ± 0.01
I	0.5520 ± 0.0003	0.247 ± 0.004	0.799 ± 0.004	1.92 ± 0.01
D	0.423 ± 0.006	0.436 ± 0.002	0.859 ± 0.008	2.01 ± 0.01
J	0.710 ± 0.008	0.61 ± 0.01	1.32 ± 0.02	3.11 ± 0.04
C	0.909 ± 0.006	0.579 ± 0.003	1.488 ± 0.009	3.54 ± 0.02
B	0.790 ± 0.006	0.74 ± 0.01	1.53 ± 0.02	3.60 ± 0.03
M	1.046 ± 0.004	0.835 ± 0.006	1.88 ± 0.01	4.46 ± 0.02
A	1.074 ± 0.003	1.293 ± 0.005	2.367 ± 0.008	5.54 ± 0.02
K	1.50 ± 0.01	1.59 ± 0.02	3.09 ± 0.03	7.24 ± 0.07

The values are expressed as mean + SD ($n = 3$).
[a)] Htyr and Tyr content corrected according to mass differences between derivatives and simple compounds. ND, non-detected.

measured concentrations of Htyr and Tyr, respectively, before and after hydrolysis.

Furthermore, our results could be important for Argentinian EVOO producers interested to adjust their production to world market that has already began to offer a new category of olive oil based on its health attribute according to the health claim approved by European Commission.

Abbreviations

Htyr, hydroxytyrosol; IOC, International Olive Council; Tyr, tyrosol.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

health claims, HPLC, hydroxytyrosol, olive oil, polyphenol

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[1] A. Scalbert, C. Manach, C. Morand, C. Rémésy, L. Jiménez, *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287.
[2] S. Martín-Peláez, M. I. Covas, F. Montserrat, A. Kušar, I. Pravst, *Mol. Nutr. Food Res*. **2013**, *57*, 760.
[3] M. T. Mijtavila, J. J. Moreno, *Biochem. Pharmacol*. **2012**, *84*, 1113.
[4] World Health Organization (**2002**). The world health report 2002, Reducing risks, promoting healthy life, 2002. http://www.who.int/whr/2002/en/Overview_E.pdf.
[5] G. Williamson, *Nutr. Bull*. **2017**, *42*, 226.
[6] Commission Regulation (EU) No 432/2012 of 16 May 2012 *Official Journal of the European Union*. **2012**, L 136/1.
[7] Commission Regulation (EU) 2015/539 of 31 March 2015, *Official Journal of the European Union*. **2015**, L 88/7.
[8] K. de la Torre-Carbot, J. L. Chavez-Servin, O. Jauregui, A. I. Castellote, R. M. Lamuela-Raventos, T. Nurmi, H. E. Poulsen, A. V. Gaddi, J. Kaikkonen, H. F. Zunft, H. Kiesewetter, M. Fito, M. I. Covas, M. C. Lopez-Sabater, *J. Nutr.* **2010**, *140*, 501.
[9] J. J. Moreno, T. Carbonell, T. Sánchez, S. Miret, M. T. Mitjavila, *J. Nutr.* **2001**, *131*, 2145.
[10] J. J. Moreno, *Free Radic. Biol. Med*. **2003**, *35*, 1073.
[11] M. Vivancos, J. J. Moreno, *Br. J. Nutr.* **2008**, *99*, 1199.
[12] N. Mulinacci, C. Giaccherini, M. Innocenti, A. Romani, F. F. Vincieri, F. Marotta, A. Mattei, *J. Sci. Food. Agric*. **2005**, *85*, 662.
[13] G. F. Montedoro, M. Servili, M. Baldioli, R. Selvaggini, E. Miniati, A. Macchioni, *J. Agric. Food Chem*. **1993**, *41*, 2228.
[14] A. Gomez-Rico, G. Fregapane, M. D. Salvador, *Food Res. Int*. **2008**, *41*, 433.
[15] M. Servili, R. Selvaggini, S. Esposto, A. Taticchi, G. Montedoro, G. Morozzi, *J. Chromatogr. A* **2004**, *1054*, 113.
[16] A. Gómez-Rico, M. D. Salvador, M. La Greca, G. Fregapane, *J. Agric. Food Chem*. **2006**, *54*, 7130.
[17] N. Romero, J. Saavedra, F. Tapia, B. Sepúlveda, R. Aparicio, *J. Sci. Food Agric*. **2016**, *96*, 583.
[18] G. Purcaro, R. Codony, L. Pizzale, C. Mariani, L. Conte, *Eur. J. Lipid Sci. Technol*. **2014**, *116*, 805.
[19] M. Ricciutelli, S. Marconi, M. C. Boarelli, G. Caprioli, G. Sagratini, R. Ballini, D. Fiorini, *J. Chromatogr. A* **2017**, *1481*, 53.
[20] International Olive Council (IOC), COI/T.20/Doc No 29 November **2009**.
[21] E. Karkoula, A. Skantzari, E. Melliou, P. Magiatis, *J. Agric. Food Chem*. **2014**, *62*, 600.
[22] C. Romero, M. Brenes, *J. Agric. Food Chem*. **2012**, *60*, 9017.
[23] A. Mastralexi, N. Nenadis, M. Z. Tsimidou, *J. Agric. Food Chem*. **2014**, *62*, 2459.
[24] P. Reboredo-Rodríguez, E. Valli, A. Bendini, G. Di Lecce, J. Simal-Gándara, T. Gallina Toschi, *Eur. J. Lipid Sci. Technol*. **2016**, *118*, 1593.
[25] N. Mulinacci, C. Giaccherini, F. Ieri, M. Innocenti, A. Romani, F. F. Vincieri, *J. Sci. Food Agric*. **2006**, *86*, 757.
[26] P. Pinelli, C. Galardia, N. Mulinacci, F. F. Vincieria, A. Cimato, A. Romania, *Food Chem*. **2003**, *80*, 331.
[27] EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), *EFSA J*. **2011**, *9*, 2.
[28] R. P. Monasterio, L. Olmo-García, A. Bajoub, A. Fernández-Gutiérrez, A. Carrasco-Pancorbo, *J. Agric. Food Chem*. **2017**, *65*, 8184.
[29] L. N. Ceci, D. Ramírez, D. F. Mussio, S. B. Mattar, A. A. Carelli, *J. Am. Oil Chem. Soc*. **2017**, *94*, 643.
[30] J. E. Pardo, M. Álvarez-Ortı, A. M. Rey, M. A. Cuesta, A. Silvestre, *Eur. J. Lipid Sci. Technol*. **2011**, *113*, 597.

