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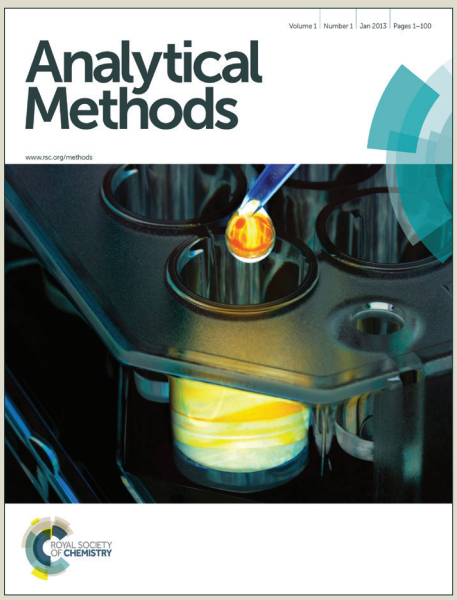

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ARTICLE TYPE

^{19}F NMR for the speciation and quantification of the OH-molecules in complex matrices

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A novel method for the speciation and the quantification of the minor OH-molecules in complex matrices in one pot experiment based on ^{19}F NMR spectroscopy is demonstrated. The method exerts high resolution and sensitivity comparable with the conventional ones and gives reliable results when directly applied on the edible oils.

Organic molecules bearing a –OH functionality (OHs), including the natural phenolic antioxidants, are widely distributed in the plant kingdom and are abundant in our diet.[1-3] The distribution of these molecules in natural foods, such as olive oil, is directly associated with the olive variety, geographical origin as well as adulteration and fruit ripening and processing.[4-6] Thus, the profile of the –OH components has been used as analytical tool for the food classification.[7] In addition, phenols are used for the oxidative stabilization, protection from formation of off-flavors and stabilization of flavors of food substances and cosmetics, whereas have been found out to exhibit positive health effects in the prevention of various diseases associated with oxidative stress such as cardiovascular and neurodegenerative diseases and cancer.[8-10] The apparent high importance of OHs has induced an immense ongoing interest in the development of novel fast analytical methods for their determination in foods as well as in cosmetics and biological materials.[11-15] Currently, HPLC is considered to be the most appropriate technique for the analysis of OHs, because of its high analytical accuracy and sensitivity.[16-18] However, it suffers from the long experimental times, the consumption of large quantities of solvents, the use of expensive standards and the time consuming pretreatment of the samples in order to enhance the separation and to concentrate the molecules under analysis.

NMR is rapidly becoming a powerful analytical tool in the hands of food chemists aiming at providing fast reliable results in relation to the adulteration, geographical and botanical discrimination and quality of foods.[19-22] However, the complex composition of these materials and the small concentration of OHs compared with the active in ^{1}H and ^{13}C NMR bulk material has imposed some serious challenges and limitations, even in the application and use of the NMR. Efforts to overcome some of the limitations imposed by ^{1}H and ^{13}C NMR spectroscopies have prompted the examination of other NMR-active nuclei. Recently a ^{31}P NMR methodology has been developed for the direct determination of OHs in foods including olive oil and wine by suitable phosphotylation of -OH groups with 2-chloro-4,4,5,5-tetramethyldioxaphospholane.[23-27] Compounds, such as diglycerides, sterols, free acids and glycerol, can be directly deteFcted in an olive oil sample, and their quantity can be accurately determined in one pot experiment by simply adding the phosphotylating agent in the sample, thus representing the fastest up to now analytical methodology for these molecules in olive oil. However, the lower receptivity of ^{31}P nucleus than ^{1}H (15 times less than ^{1}H) and its large relaxation times (5 - 10 s) result in low sensitivity. The low sensitivity can be compensated by longer acquisition times. However, even at high resolution NMR instruments (500 MHz) other minor OHs such as α-tocopherol and tyrosols cannot be directly determined but only after the minor components have been concentrated.

In this work, we like to introduce a new ^{19}F NMR method based on the fluoro-labelling of –OH chemical group of the compounds by suitable fluorine reagent for the quantitative determination of OHs in edible oils. The 100% natural abundance of the ^{19}F nucleus, the high gyromagnetic ratio and the small relaxation times (0.5-2 s) make ^{19}F NMR sensitivity nearly the same as that of a proton. Its chemical shift extends over a wide range providing adequate signal dispersion that reduce signal overlap and aid interpretation. Another advantage of using the ^{19}F nucleus as probe in the NMR measurements is the absence of fluorine in most analytical samples of interest such as coal, petroleum, wood, cosmetics, food and biological samples, thus, the signal exclusively represents the products of the fluoro-labeling reaction.[28-37] Trifluoroacetic anhydride has been chosen as the reagent for the –OH derivatization because is easy to handle, and reacts selectively in high yields with the organic functional group(s) of interest. The ^{19}F NMR chemical shifts of the trifluoroacetate derivatives of OHs are dependent upon the chemical environment of –OH providing important structural information for the targeted phenols/alcohols [Fig. 1].[38, 39]

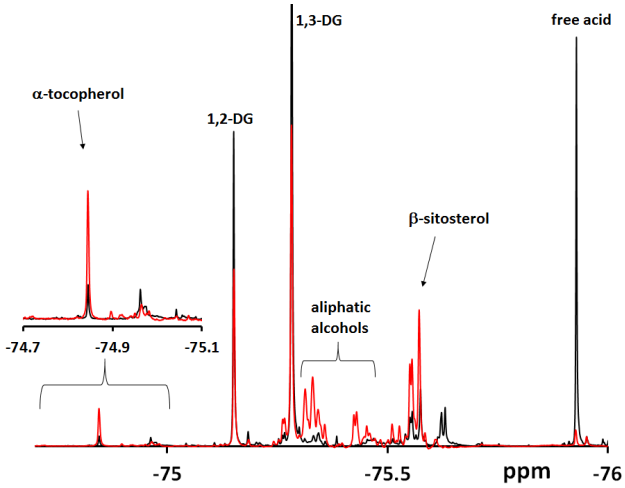
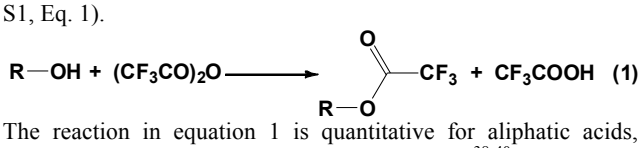


Fig.1 ^{19}F NMR spectrum at 470.5 MHz of virgin olive oil (black line) and sunflower oil (red line) both treated with trifluroacetic anhydrite according to the procedure **B**. The two oils can be easily discriminated from the peaks of the aliphatic alcohols which are present in high quantities only in sunflower oil. DG=diglycerides.

The presence of the three equivalent fluorine nuclei, in the trifluoromethyl moiety used for the OHs' derivatization, has an additive effect in the intensity of the ^{19}F NMR peaks thus resulting in high sensitivity. The high sensitivity permits the direct determination of OHs, even minor species such as α-tocopherol, in edible oils.

Preparation of the trifluoroacetates is easily accomplished by adding 3-5 times molar excess of $(CF_3CO)_2O$ in solutions of OHs in $CDCl_3$ or CD_2Cl_2 (see experimental in supplementary material S1, Eq. 1).



The reaction in equation 1 is quantitative for aliphatic acids, primary, secondary and cyclic aliphatic alcohols.[38-40] On contrary, the esterification of the aromatic phenols and sterically hindered alcohols is partial and slow even at higher excess of the anhydrite. Addition of small quantities of base (pyridine or tributylamine) (base/anhydrite ~ 0.3) catalyzes the reaction resulting in the fast and quantitative esterification of all the OHs under study.

The ^{19}F chemical shift data (Table S1) for the trifluoroacetate derivatives of OHs cover a range of ~2 ppm, exhibiting the following order, from low to high field: phenols > aliphatic polyalcohols > benzyl alcohols > primary alcohols > 6-membered cyclic secondary alchohols > secondary alcohols > aromatic acids > aliphatic acids > tertiary alcohols. The ^{19}F shielding constant depends much more on the chemical environment, and the spectra are simpler than the spectra of protons in ^{1}H NMR, resulting in the facile assignment of the peaks in the mixtures of OHs. For example, the α-, β-, γ- and δ- tocopherols from a rice extract can be easily distinguished from each other as shown in Fig. 2. The assignment of the peaks was done with spike experiments by adding either α- or γ- or δ- pure tocopherol in the extract. The quantification of OHs was validated by the construction of the calibration curves in $CDCl_3$. Solutions of known varying concentrations of OHs (between 1.4 – 15 mM), such as α-tocopherol, β-sitosterol, eicosanol, homovanillyl alcohol, 1,2- and 1,3- diglycerols (1,2- and 1,3-DG), 2,2'-dimethylcyclohexanol and oleic acid, reacted with trifluoroacetic anhydride in $CDCl_3$ or CD_2Cl_2 in the presence of internal standard (*tert*-butanol). The quantification of OHs also was validated by the construction of the calibration curves on samples containing olive oil. The graphs of the calculated from ^{19}F NMR *vs* the added concentrations were linear proving that the esterification of the OHs is quantitative and independent on other substances present in the oil samples (Fig. S1-S5). The concentration of the esterified -OHs were estimated from the ^{19}F peak integrals related to the integral of the peak of the internal standard. The calculated concentration is linearly related with the introduced analyte concentration and the linear regression analysis on the data gave correlation coefficient better than 0.998. Calibration curves of the mixtures of OHs gave similar results.

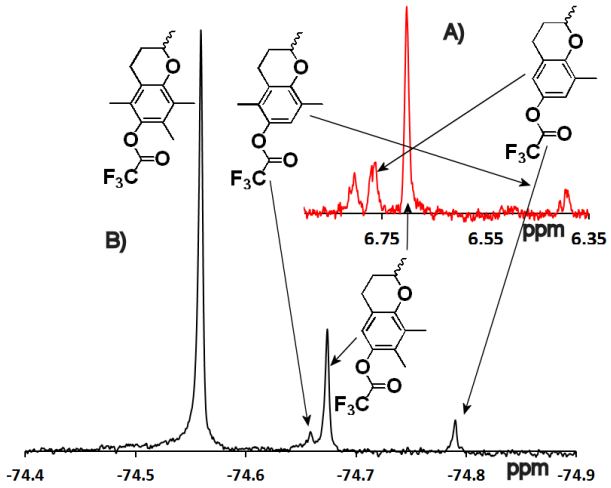


Fig.2 ^{1}H NMR (500 MHz) (red line, upper spectrum) of the aromatic region of a $CDCl_3$ solution of a standard mixture of tocopherols provided by Eastman Chemical from rice extract, and the ^{19}F NMR (470.5 MHz) (black line, lower spectrum) of the same extract treated with excess of $(CF_3CO_2)_2O$ (5 equivalents). The assignment of the peaks was done with spike experiments by adding either α- or γ- or δ- pure tocopherol in the extract.

Samples of ~0.5 g of edible oils from various sources were allowed to react with excess of trifluoroacetic anhydrite (80-150 μL of 1.35 M solution in $CDCl_3$), with and without the presence of small quantity of base (pyridine or tributylamine 25-50 μL of 1.35 M solution in $CDCl_3$) and an accurate quantity of the standard compound (50.0 μL of 0.140 M, cyclohexanol, or 2-pentanol or *tert*-butanol) (S1). 2-pentanol gave a non-overlapping signal and was preferred for the experiments in oils than the other standards. Although *tert*-butanol gave a non-overlapping signal as well, it requires pyridine for quantitative reaction with the trifluoroacetic anhydrite. Cyclohexanol is a known standard from the literature,[23] however gave a ^{19}F signal close to the chemical shift of β-sitosterol. The spectra of the samples without the base gave peaks assigned to the trifluoroacetate derivatives of the OHs present in the oils, one peak from the standard compound, one peak originated from the free trifluoroacetic acid and one peak from the trifluoroacetic anhydrite [Fig. 3(A)]. The presence of the peak of the anhydrite consists a good indicator showing whether the anhydrite is rather in excess than completely consumed by the reaction with OHs and the water in oils. The identity of the peaks was determined by spike experiments. The spectra of the samples with the base were similar with those of the samples containing no base with the exception of the increase of the intensity of the esterified phenols and the broadening of the trifluoroacetic acid and anhydride peaks due to the chemical exchange described by the equation 2.

$$CF^*_3COO^- + (CF_3CO)_2O \rightleftharpoons CF_3COO^- + (CF_3CO)O(CF^*_3CO) \quad (2)$$

The rate of the reaction 2 is increased with additional base resulting in the coalescence of the two peaks originated from the fast chemical exchange of the acid and the anhydrite. The peaks of the acid and the anhydrite are very broad covering a large part of the spectra [Fig. 3(B)] making the quantification of the peaks difficult in this region. Thus, the OHs were quantified by proper curve fitting of the peaks in the spectra (Fig. S6-S7). At higher concentrations of base, the chemical exchange is very fast, the two peaks turned to one sharp peak, however the peaks assigned to the mixed free fatty acids - trifluoroacetic anhydrite [(RCO)-O-($OCCF_3$)] became broad due to the chemical exchange shown in equation 3 and additional peaks were appeared due to the formation of adducts of trifluoroacetic anhydrite with the base.

$$CF^*_3COO^- + (CF_3CO)O(RCO) \rightleftharpoons CF_3COO^- + (CF^*_3CO)O(RCO) \quad (3)$$

All the complications caused by the relative large quantity of the trifluoroacetic acid and the anhydrite in the under study oil samples were diminished by washing the solutions twice with small quantities of water. The trifluoroacetic and the mixed trifluoroacetic – free acid anhydrites hydrolyzed immediately after the addition of the water and the most of the free trifluoroacetic acid was transferred from the organic to the aqueous phase, and removed. The hydrolysis of the anhydrites was also used as a tool for the correct assignment of the peaks separating the free acid anhydrites from the alcoholic and phenolic trifluoroacetate esters. In addition, the OHs containing both –OH and –COOH functional groups can be assigned with the hydrolysis of the (RCO)-O-($OCCF_3$) anhydrites, which results in shift of the R–O-($OCCF_3$) trifluoroacetate esters peaks [Fig. 3(C)]. The comparison of the integrals of trifluoroacetate esters of OHs between the spectra of the samples with and without base and after the addition of water revealed that the esters remained intact after the anhydrites hydrolysis.

Procedure (B) is proposed for complete quantification of the OHs in olive oil. Procedure (A) can only be used for the measurement of the nonaromatic OHs of oil as far 2-pentanol is used as internal quantitative standard. Although procedure (C) leads to the same quantitative results as (B), cannot be used for the determination of free fatty acids and requires additional treatment and time.

The LOD and LOQ values were calculated for all the studied OHs from the standard solutions and were comparable to the values calculated for the same OHs species in olive oils solutions. For example LOD values for α-tocopherol in $CHCl_3$ solutions and in the olive oil solution were found 16 mg/L and 15 mg/Kg respectively, while the LOQ values were 49 and 47 mg/Kg respectively. For comparison with the phospholane-derivatization method,[41] LOD values for OHs (α-tocopherol, tyrosol, DGs and β-sitosterol) were also calculated based on the S/N ratio for 32 scans spectra and were found to be in the range 2.4 to 7.5 mg/Kg. LOD from S/N ratio calculated by the phospholane-derivatization method for the polar extraction of 35 g of olive oil has been reported to be in the range 0.26 to 0.86 mg/Kg for spectra of the same number of scans. Extrapolation of the phospholan method to the amounts used for the ^{19}F NMR derivatization method results in LOD values in the range 18.2 to 60.2 mg/Kg.

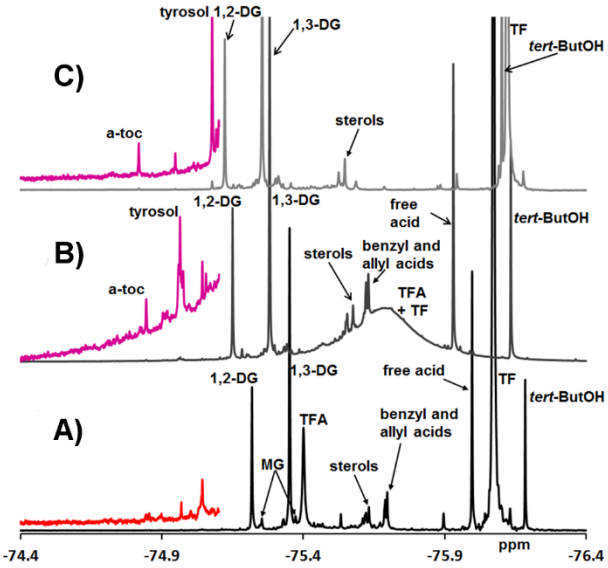


Fig.3 ^{19}F NMR of a tocopherol mixture of a $CDCl_3$ solution of a rice extract treated: A) with excess of $(CF_3CO_2)_2O$ (0.20 mmols), B) excess of $(CF_3CO_2)_2O$ (0.20 mmols) and pyridine (0.067 mmoles), C) excess of $(CF_3CO_2)_2O$ (0.20 mmols) and pyridine (0.067 mmoles) and then washed with water to remove the excess of TFA and TF. Spectra acquired in 32 scans at 470.5 MHz. The chemical shift region -74.40 up to -75.10 ppm has been magnified in order to be seen because of the small concentration of phenolics in olive oil compared to the high concentrations of sterols and 1,2- and 1,3-DG. TFA stands for Trifluoroacetic anhydrite and TF for Trifluoroacetic acid.

The quantities of a-tocopherol, 1,2- and 1,3- DG, sterols and free fatty acids in three oil samples were also analyzed with conventional methods by independent analytical laboratories. The α-tocopherol analysis was performed by HPLC, and the diglycerols and sterols analyses by GC Chromatography. The results of the analyses were compared with those obtained by the ^{19}F NMR methodology (Table S2-S3). The data shows that the ^{19}F NMR method is excellent correlated with the conventional methods.

Moreover, the ^{19}F NMR spectra of the trifluoroacetic anhydrite treated edible oils provide a fast and detailed fingerprint of the oil components. Since OHs consist primary markers for the identification of the different cultivar, the method will excellently serve for the quick discrimination between the edible oils. For example, the discrimination between the extra virgin olive and Sunflower oil is mainly based on the profile and the quantity of fatty alcohols [Fig.1].

Conclusions

Concluding, in this manuscript we have illustrated the fastest ever reported method for the quantitative determination of OHs in edible oils, without any pretreatment. This method for the OHs analyses is the cheapest reported up to date and can simultaneously analyze several phenols, alcohols and organic acids. The ^{19}F NMR spectra of the derivatized oil give simultaneously the quantities of α-tocopherol, tyrosols, diglycerides, monoglycerides, sterols and free acids in a very short time diminishing the high cost of the instrumentation. Low frequency and subsequently lower cost instruments such as 300 MHz NMR are sufficient for this analysis. The new method based on ^{19}F NMR is more sensitive than the phospholane-derivatization method with 8 times smaller LOD value for

phenols. In addition, the total conversion of the OHs to the respective trifluoroacetate derivatives enables full recovery of the method. Because of the high selectivity, the method can be used for the structural characterization of the environment around the –OH groups and the detection of new unknown species. The dispersion of the ^{19}F NMR peaks over a large range of chemical shifts as well as the unambiguous assignment of the different OH groups emerge the ^{19}F NMR spectroscopy as a very powerful tool for the quantification of OHs in edible oil and other food matrixes, as well in reaction mixtures. Both the simplicity in the interpretation of the ^{19}F NMR spectra and the facile experimental procedure promote the use of NMR as analytical tool not only in chemical research but also in industry, public and private analytical laboratories.

To the best of our knowledge, this is the first study detailing edible oil derivatization followed by ^{19}F NMR spectroscopy for the quantitative analysis of minor OHs species.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [S1, Figures S1-S7, Tables S1-S3]. See DOI: 10.1039/b000000x/

1. S. A. Heleno, A. Martins, M. J. R. P. Queiroz and I. C. F. R. Ferreira, *Food Chemistry*, 2015, **173**, 501-513.
2. L. Liu, C. Jin and Y. Zhang, *RSC Advances*, 2014, **4**, 2879-2891.
3. D. Ryan and K. Robards, *Analyst*, 1998, **123**, 31R-44R.
4. F. Longobardi, A. Ventrella, G. Casiello, D. Sacco, M. Tasioula-Margari, A. K. Kiritsakis and M. G. Kontominas, *Food Chemistry*, 2012, **133**, 169-175.
5. I. F. F. Benzie and S. W. Choi, in *Advances in Food and Nutrition Research*, 2014, vol. 71, pp. 1-53.
6. T. S. Villani, W. Reichert, M. G. Ferruzzi, G. M. Pasinetti, J. E. Simon and Q. Wu, *Food Chemistry*, 2015, **170**, 271-280.
7. A. Khoddami, M. Wilkes and T. Roberts, *Molecules*, 2013, **18**, 2328-2375.
8. R. Mateos, A. Madrona, G. Pereira-Caro, V. Domínguez, R. M. Cert, J. Parrado, B. Sarriá, L. Bravo and J. L. Espartero, *Food Chemistry*, 2015, **173**, 313-320.
9. F. Rubio-Senent, B. De Roos, G. Duthie, J. Fernández-Bolaños and G. Rodríguez-Gutiérrez, *European Journal of Nutrition*, 2014.
10. R.-M. Valls, M. Farràs, M. Suárez, S. Fernández-Castillejo, M. Fitó, V. Konstantinidou, F. Fuentes, J. López-Miranda, M. Giralt, M.-I. Covas, M.-J. Motilva and R. Solà, *Food Chemistry*, 2015, **167**, 30-35.
11. I. Tarascou, J. M. Souquet, J. P. Mazauric, S. Carrillo, S. Coq, F. Canon, H. Fulcrand and V. Cheynier, *Archives of Biochemistry and Biophysics*, 2010, **501**, 16-22.
12. C. M. Ajila, S. K. Brar, M. Verma, R. D. Tyagi, S. Godbout and J. R. Valéro, *Critical Reviews in Biotechnology*, 2011, **31**, 227-249.
13. E. N. Frankel, *Journal of Agricultural and Food Chemistry*, 2011, **59**, 785-792.
14. V. Rastija, *Mini-Reviews in Medicinal Chemistry*, 2011, **11**, 1256-1267.
15. K. M. Kalili and A. De Villiers, *Journal of Separation Science*, 2011, **34**, 854-876.
16. R. Mateos, J. L. Espartero, M. Trujillo, J. J. Ríos, M. León-Camacho, F. Alcudia and A. Cert, *Journal of Agricultural and Food Chemistry*, 2001, **49**, 2185-2192.
17. K. Robards, *Journal of Chromatography A*, 2003, **1000**, 657-691.
18. R. J. Robbins, *Journal of Agricultural and Food Chemistry*, 2003, **51**, 2866-2887.
19. R. Popescu, D. Costinel, O. R. Dinca, A. Marinescu, I. Stefanescu and R. E. Ionete, *Food Control*, 2015, **48**, 84-90.
20. L. Mannina, M. D'Imperio, D. Capitani, S. Rezzi, C. Guillou, T. Mavromoustakos, M. D. M. Vilchez, A. H. Fernández, F. Thomas and R. Aparicio, *Journal of Agricultural and Food Chemistry*, 2009, **57**, 11550-11556.
21. G. Le Gall and I. J. Colquhoun, in *Food Authenticity and Traceability*, 2013, pp. 131-155.
22. R. Lamanna, in *Annual Reports on NMR Spectroscopy*, 2013, vol. 80, pp. 239-291.
23. A. Spyros and P. Dais, *Journal of Agricultural and Food Chemistry*, 2000, **48**, 802-805.
24. G. Vigli, A. Philippidis, A. Spyros and P. Dais, *Journal of Agricultural and Food Chemistry*, 2003, **51**, 5715-5722.
25. A. Spyros and P. Dais, *Progress in Nuclear Magnetic Resonance Spectroscopy*, 2009, **54**, 195-207.
26. P. Dais and E. Hatzakis, *Analytica Chimica Acta*, 2013, **765**, 1-27.
27. C. Lucas-Torres, Á. Pérez, B. Cabañas and A. Moreno, *Food Chemistry*, 2014, **165**, 21-28.
28. S. André, F. J. Cañada, T. C. Shiao, L. Largartera, T. Diercks, M. Bergeron-Brlek, K. El Biari, A. Papadopoulos, J. P. Ribeiro, M. Touaibia, D. Solís, M. Menéndez, J. Jiménez-Barbero, R. Roy and H. J. Gabius, *European Journal of Organic Chemistry*, 2012, 4354-4364.
29. M. Braitsch, H. Kählig, G. Kontaxis, M. Fischer, T. Kawada, R. Konrat and W. Schmid, *Beilstein Journal of Organic Chemistry*, 2012, **8**, 448-455.
30. P. B. Crowley, C. Kyne and W. B. Monteith, *Chemical Communications*, 2012, **48**, 10681-10683.
31. K. Fauster, C. Kreutz and R. Micura, *Angewandte Chemie - International Edition*, 2012, **51**, 13080-13084.
32. F. Michel, S. Hamman, F. Thomas, C. Philouze, I. Gautier-Luneau and J. L. Pierre, *Chemical Communications*, 2006, 4122-4124.
33. A. Moghimi, I. Omrani, M. R. Nabid and M. Mahmoodi, *European Polymer Journal*, 2013, **49**, 228-234.
34. M. Sarkouhi, J. Hassan and M. Shamsipur, *Applied Magnetic Resonance*, 2012, **43**, 377-384.
35. A. N. Tkachenko, P. K. Mykhailiuk, S. Afonin, D. S. Radchenko, V. S. Kubyshkin, A. S. Ulrich and I. V. Komarov, *Angewandte Chemie - International Edition*, 2013, **52**, 1486-1489.
36. B. C. Ahvazi, C. Crestini and D. S. Argyropoulos, *Journal of Agricultural and Food Chemistry*, 1999, **47**, 190-201.
37. F. Huang, S. Pan, Y. Pu, H. Ben and A. J. Ragauskas, *RSC Advances*, 2014, **4**, 17743-17747.
38. S. L. Manatt, *Journal of the American Chemical Society*, 1966, **88**, 1323-1324.
39. P. Sleevi, *Analytical Chemistry*, 1979, **51**, 1931-1934.
40. H. J. Schneider, G. Jung, E. Breitmaier and W. Voelter, *Tetrahedron*, 1970, **26**, 5369-5376.
41. P. Dais and A. Spyros, *Magnetic Resonance in Chemistry*, 2007, **45**, 367-377.