Research Article

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A novel fluorescent-based HPLC method for determination of urolithins

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ABSTRACT: Urolithins are the bioavailable metabolites of ellagitannins, abundantly available in dietaries routinely consumed. The number of research studies growingly enlarges on the beneficial effects of these natural chemicals on human health. From this perspective, one aspect of scientific interest in urolithins covers the development of techniques for their qualitative and quantitative analysis in diverse samples. UV-based and tandem-mass spectroscopic analyses have been the major ones used and applied so far. However, the fluorescent characteristics of these molecules have not received sufficient attention. Within the scope of this research study, major urolithins (Urolithin A and B) concomitant to their methyl ether metabolites have been synthesized and their quantitative analysis has been investigated via an HPLC system connected to fluorescence detection. The results displayed the superior and comparable characteristics of the methodology developed against the previous techniques.

KEYWORDS: Urolithin A; Urolithin B; Urolithin methyl ethers; Fluorescence; HPLC; quantification.

1. INTRODUCTION

Urolithins, hydroxylated benzo[c]chromen-6-one derivatives, are ellagitannin metabolism products. Many living things, including humankind, are routinely exposed to these ellagitannins (e.g., punicalagin, punicalin) via different dietary sources, particularly including pomegranate, nuts, and berries [1-2]. These relative macromolecules are subject to intensive gastrointestinal tract biotransformation pathways leading to the formation of ellagic acid which also has very poor bioavailability [3]. Ellagic acid is further subject to metabolism, catalyzed by gut microbiota, to generate urolithins. Among them, the urolithin A (3,8-dihydroxybenzo[c]chromen-6-one) and the urolithin B (3-hydroxybenzo[c]-chromen-6-one) are the major ones formed [4-5]. Urolithins are bioavailable and therefore they are acknowledged as the biomarkers of ellagitannin metabolism [6].

Regarding the beneficial effects of ellagitannin-containing foods on diverse biological systems, many studies have been conducted on their antioxidant, antimicrobial, anti-inflammatory, and even anticancer effects [3, 7-8]. Since ellagitannins and ellagic acid suffer from absorption following exposure, the scientific interest is more focused on their bioavailable metabolites, urolithins (particularly urolithin A and B), in the last two decades. Indeed, the research studies are even extrapolated to the possible employment of these compounds in the treatment of central nervous system diseases [9-10].

One of the distinct features of urolithins is their fluorescent properties [11]. Our previous studies showed the Iron (III) selective on-off fluorescence probe characteristics of the main urolithins and their methyl ether metabolites [12]. These studies were also extrapolated to the substituent effects and some derivatives of these compounds were also shown to display fluorescence enhancement properties in the presence of some metals [12-13].

One of the scientific interests in urolithins is related to the development of qualification and quantification techniques in different samples [14-15]. Urolithins are UV-active compounds. Therefore, UV-spectrophotometric and HPLC methodologies attached to UV-, or DAD-detectors have been suggested as significant systems for urolithin analysis [16-17]. Particularly, the scientific improvement in mass spectroscopy and the related tandem systems such as LC-MS/MS also yielded very efficient methodologies to characterize many urolithin metabolites [18-19].

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As appreciated, fluorescent techniques are very sensitive methodologies; therefore, the qualitative and quantitative analysis of fluorescent molecules have certain advantages with respect to the separation, qualification, and quantification perspectives [20]. Within this research study, it was aimed to develop a methodology employing an HPLC system connected to a fluorescent detector to sensitively qualify and quantify the major urolithins (i.e., Urolithin A, and B, abbreviated as URO-A, and URO-B, respectively within the manuscript) and their methyl ether metabolites (i.e., URO-A-M, and URO-B-M), all available in systemic circulation upon exposure to ellagitannin rich diet (Figure 1). Besides the validation studies on the method developed, the comparison of the new methodology was also questioned with respect to the features of already published and used techniques.

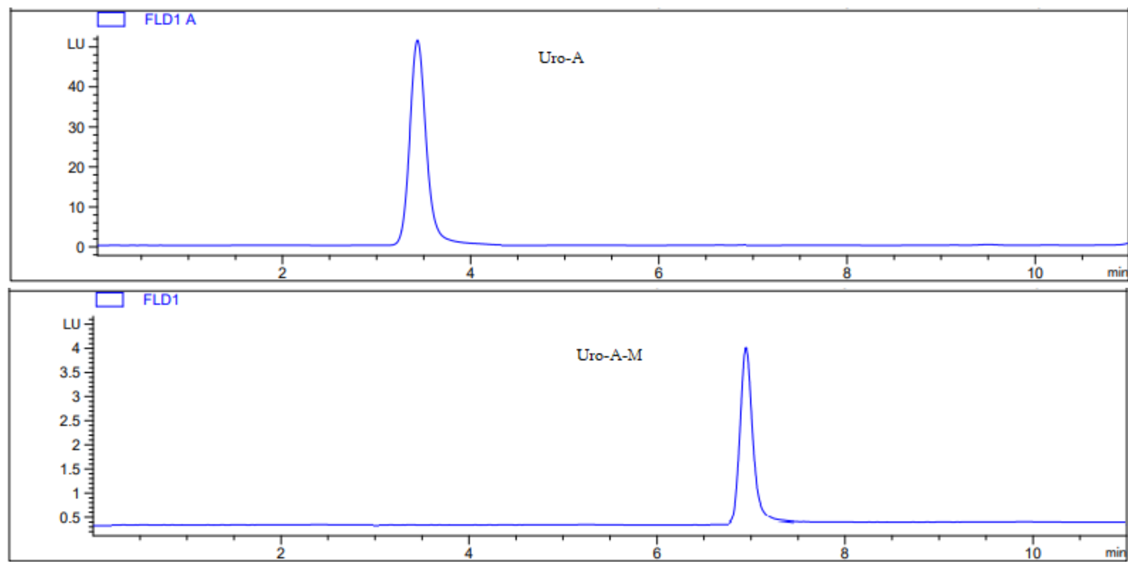
Urolithin A (URO-A) | Urolithin B (URO-B) | Urolithin A Methyl ether (URO-A-M) | Urolithin B Methyl ether (URO-B-M)

Figure 1. The title urolithin molecules.

2. RESULTS

The synthesis of the title molecules has been achieved through previously published procedures. The yields and the spectral analysis were all in parallel with the previous data [12-13]. In order to develop an efficient methodology for the HPLC monitoring of the substances, the work that has been generated in our previous studies was improved [8]. Accordingly, the gradient profile employing only water-formic acid up to 90% through reaching 27 min resulted in the appearance of the peaks of title molecules in more than 30 min. A fast gradient system in which the time is narrowed to reach to specified concentration did not aid in shortening the retention time. The isocratic system employing 30% solvent A and 70% solvent B gave the best results in which retention times were less than 10 min. The monitoring was achieved by employing both DAD and fluorescence detectors to make additional visual comparisons.

The specificity of the methodology was expressed with no interference in the standard chromatogram upon the injection of the blank solution. For each title compound, 5 different concentrations were used to test the method for linearity. The regression analyses were conducted in parallel to the calculation of the least square methods correlation coefficient, slope, and intercept values. The chromatograms, displaying the retention of the title urolithins with fluorescence detection according to the specificity assays, and the related regression curves, obtained through linearity studies, are shown in Figures 2 and 3.



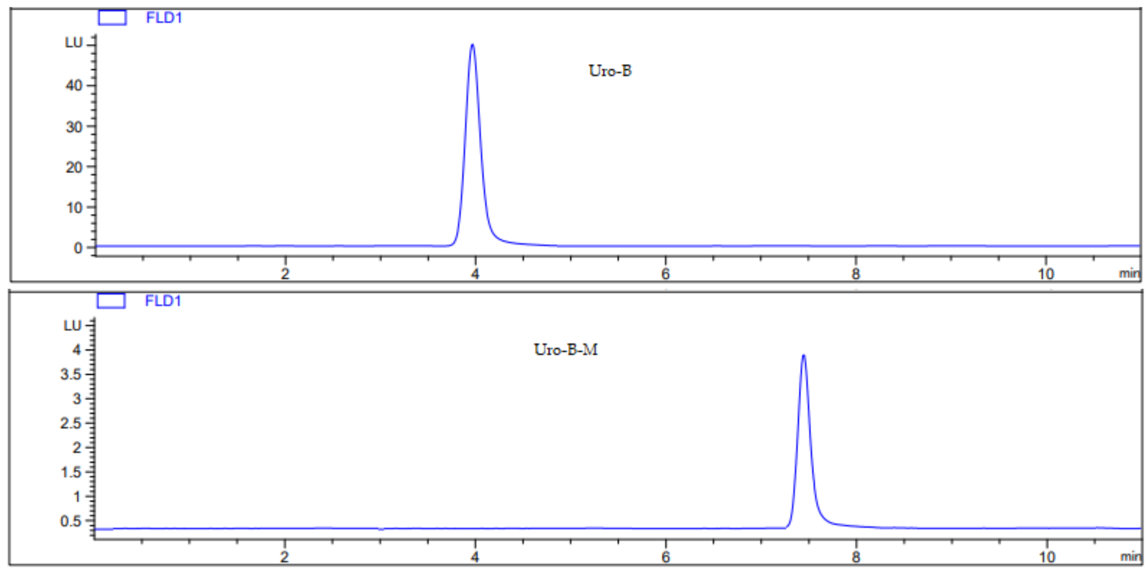
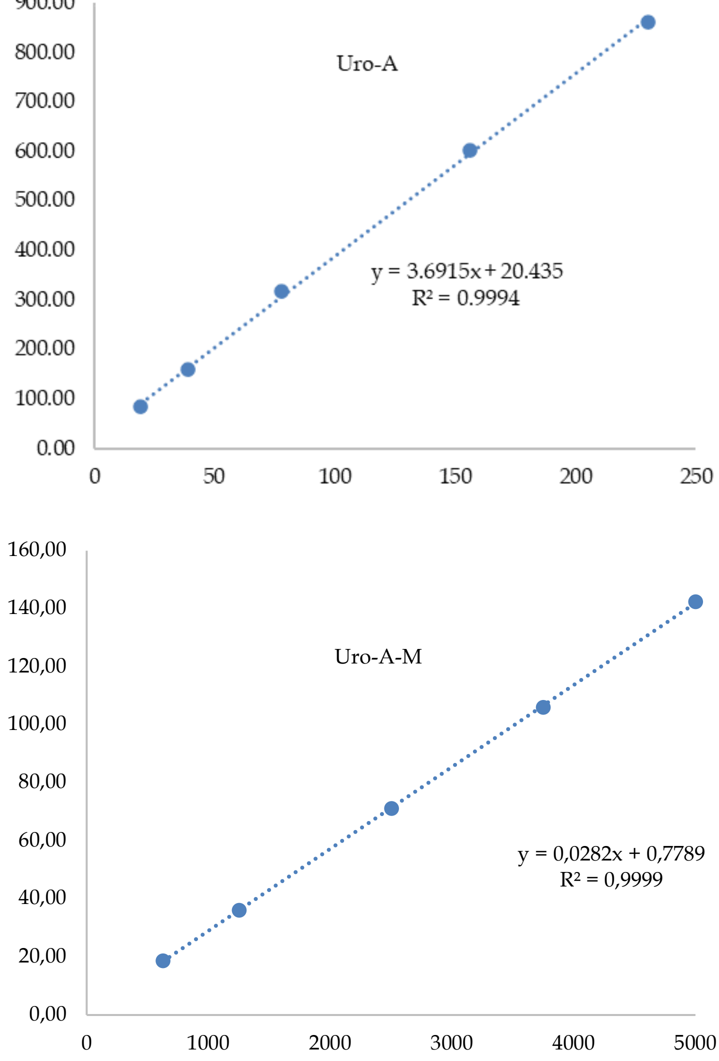


Figure 2. HPLC chromatograms upon fluorescence detection and the specificity of the method developed.



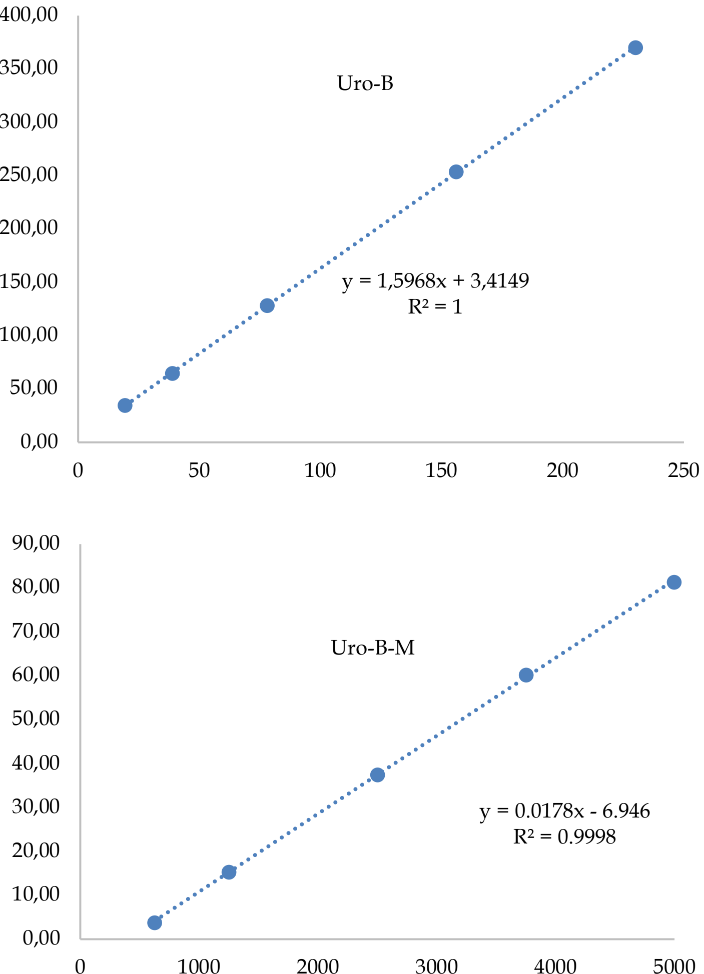


Figure 3. The results of the linearity studies (Fluorescence intensity vs concentration (ng/mL)).

Accordingly, the linearity for each title molecule with the developed fluorescence detection was established with the coefficient of determination obtained (i.e., each was higher than 0.9990). It is of significance to also state that depending on the substituent effect on benzo[c]chromen-6-one system, the general fluorescence intensities obtained for Urolithin A and B were found higher in comparison to the fluorescence intensities obtained for their methyl ether derivatives which were in parallel with our previous observations [12-13, 21]. The specificity studies apparently displayed the visual priority for the fluorescence detectors over DAD detectors. As seen in Figure 4, for instance, there has been no observation of urolithin B peak at 30ng/mL through the employment of a DAD detector (set to 254 and 280nm wavelengths) whereas, it became apparent at the same concentration via the aid of fluorescence detection under the experimental conditions developed.

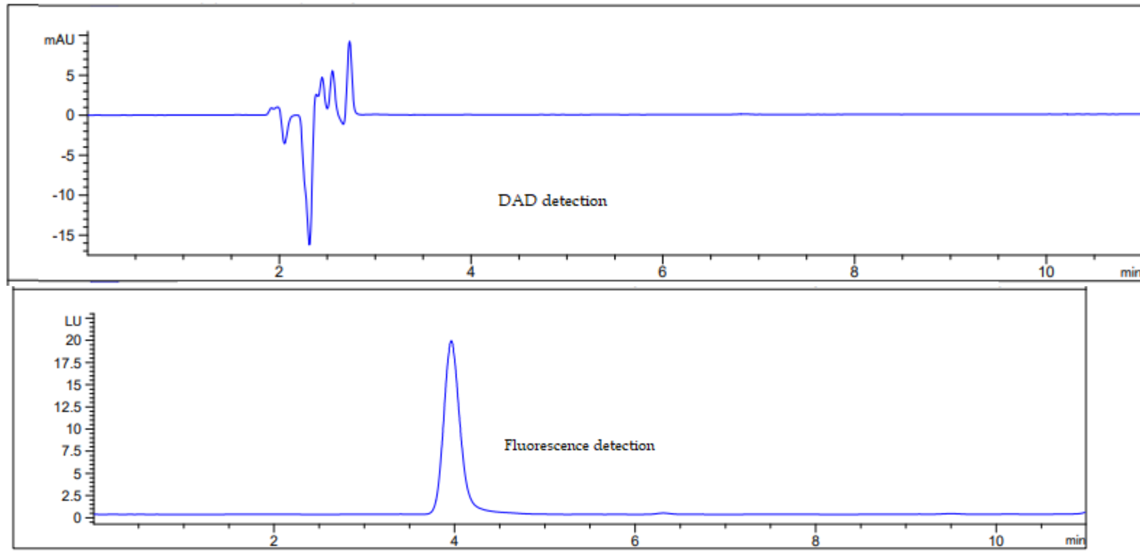


Figure 4. The superiority of the fluorescence detection method developed over DAD detection (urolithin B at 30 ng/ml)

Precision and recovery studies have also been conducted. Known amounts of samples were weighed and employed to calculate the percentages of recovery in 3 different measurements. The results are expressed in Table 1. Accordingly, percent average recoveries were all found in 98.9-100.7 % as also expressed by their relative standard deviation. Intra-day and inter-day variations have also been assessed in precision experiments. As shown in table 1, the results indicated the relative standard deviations (%).

Table 1. The results of the recovery studies

Compound	%	Added amount	Recovered amount	% Recovery	% Average
Uro-A	50	39	39 ± 1.96	100 ± 0.002	100.04
	100	78	78 ± 5.34	100 ± 0.005	100.56
	150	156	156 ± 5.61	100 ± 0.006	99.55
Uro-A-M	50	1250	1250 ± 12.81	100 ± 0.003	99.48
	100	2500	2500 ± 13.96	100 ± 0.004	99.86
	150	3750	3750 ± 13.72	100 ± 0.004	99.75
Uro-B	50	39	39 ± 3.85	100 ± 0.004	100.26
	100	78	78 ± 3.92	100 ± 0.004	100.21
	150	156	156 ± 3.75	100 ± 0.004	99.75
Uro-B-M	50	1250	1250 ± 11.10	100 ± 0.001	100.47
	100	2500	2500 ± 10.82	100 ± 0.001	98.93
	150	3750	3750 ± 11.00	100 ± 0.001	100.66

The precision of the methodology was assessed through the analysis of multiple injections of urolithin replicates and the range was expressed in %RSD values. Accordingly, both intraday and inter-day measurements yielded a range of 0.005-0.018 %RSD (Table 2).

Table 2. Precision of the methodology.

Compound	Intraday precision Peak Area (%RSD)	Inter-day precision Peak Area (%RSD)
Uro-A	617.61 ± 6.345 (0.01)	603.17 ± 3.572 (0.006)
Uro-A-M	71.14 ± 0.416 (0.006)	66.06 ± 0.482 (0.007)
Uro-B	253.73 ± 4.479 (0.018)	254.51 ± 2.454 (0.01)
Uro-B-M	37.47 ± 0.182 (0.005)	37.44 ± 0.282 (0.008)

The fluorescence-based developed methodology has been further analyzed to evaluate the LOD (limit of detection) and LOQ (limit of quantification) of the title urolithins analyzed. LOQ has been established as the lowest concentration that could be measured in the 80-120% accuracy levels. In the technical description via the employment of HPLC, LOD, and LOQ are the, respectively, three-, and ten-times signal to noise ratio in diluted standard applications. With respect to the LOD and LOQ analysis of the urolithins, the results obtained are shown in Table 3.

Table 3. LOD and LOQ values of the title urolithins

Compound	LOD (ng/mL) FD	LOQ (ng/mL) FD	LOD (ng/mL) DAD	LOQ (ng/mL) DAD
Uro-A	2.07	6.26	70.42	213.39
Uro-A-M	146.75	444.69	641.07	1942.63
Uro-B	3.48	10.56	90.48	274.18
Uro-B-M	175.53	531.92	741.25	2246.59

Accordingly, both LOD and LOQ values obtained under fluorescence detection methodology conditions for the title molecules analyzed yielded quite lower results in comparison to the DAD detection-based values. LODs and LOQs of URO-A and URO-B were also found less than the values for their corresponding methyl ether derivatives. Overall, it was apparently observed that the fluorescence detection methodology for the major urolithins analyzed has been shown to possess superiority over the UV-based detection and quantification techniques.

3. DISCUSSION

Diverse studies have been carried out so far for the qualitative and quantitative analysis of urolithin derivatives. It is known that urolithins are gastrointestinal metabolites of ellagitannin-containing dietaries. Therefore, urolithins are bioavailable biotransformation products. The studies conducted in mammalians, including humankind, clearly pointed out that the most abundant urolithins in plasma cover the urolithins B and A, their phase II conjugates (sulfate and glucuronide conjugates), and their methyl ethers [22-23]. Although limited attention has been focused on the methyl ether derivatives, previous studies displayed that the central nervous system penetration of urolithins has been shown to be possible via the function of catechol-O-methyltransferase enzyme function on these phenolic compounds, since the methyl ether metabolites are the ones identified in CNS [24-25]. From this perspective, this study, on the one hand, displays the efficient employment of the methodology for the monitoring and quantification of urolithin A, urolithin B, and their corresponding methyl ether metabolites. Future studies are warranted to see the availability of the experimental procedure provided for the conjugates of urolithins rather than methyl conjugation.

One aspect of the research also displayed the advantages part of fluorescence-based quantification. Indeed, the LOD and LOQs obtained were all found less than the ones for pure UV detection. Previous studies indicated the concentrations of diverse urolithins in plasma can reach up to 100μM levels upon exposure to an ellagitannin-rich diet [14-26]. The LOD and LOQ concentrations via the fluorescence technique displayed in this research study pointed out the very low nanomolar levels that can be either detected or quantified. Obviously, sophisticated techniques such as the employment of LC/MS and LC/MS-MS systems are efficiently utilized in xenobiotic metabolism studies to increase the sensitivity of diverse compound detection and quantification. Although these instruments provide certain advantages, it is well-known that these techniques require the presence of instruments, know-how technology, and considerable funding. From this perspective, fluorescence techniques wherever possible generate sensitive, cheap, and easier methodologies. Therefore, the methodology described in this study apparently introduces that fluorescence detection and quantification via an HPLC methodology using a fluorescence detector is a superior and comparable methodology, respectively against UV and MS/MS systems, for urolithin compounds possessing fluorescent properties. It is important to note that the retention times obtained for the title urolithins employed are relatively closer. This might create handicap in alternative applications particularly on biological samples. Therefore, improvements should be warranted in future studies.

4. CONCLUSION

UV and tandem-mass systems are efficiently utilized in the quantification of diverse chemicals including the studies conducted on the qualitative and quantitative perspectives of xenobiotic metabolism in humans. The analysis of the fate of urolithins in mammalians and in human have attracted attention, particularly in the last two decades. The majority of the studies are based on UV detection, whereas tandem systems are also employed. It is known that major urolithins (urolithin A and B) are fluorescent compounds. Regarding this feature, also present in their corresponding methyl ethers, this study has provided an alternative perspective on the qualification and quantification of the title urolithins via the use of fluorescence technique combined with HPLC. The methodology described has been proven to be superior to detection via DAD detectors. On the other hand, the LOD and LOQ values calculated were also found promising in order to conduct fluorescence-based experiments on the identification of urolithin levels in living things upon exposure to ellagic acid-generating xenobiotics.

5. MATERIALS AND METHODS

5.1 Chemicals

Resorcinol, 2-bromobenzoic acid, 5-hydroxy-2-bromobenzoic acid, copper sulfate, sodium hydride, and methyl iodide were obtained from Sigma Aldrich and they were all reagent grade. Dimethyl sulfoxide, acetonitrile, and formic acid were HPLC grade and they were purchased from local vendors. IA9000 Series Melting Point Apparatus was used for melting point determinations. A Bruker-400 NMR spectrometer with tetramethylsilane (TMS) as an internal standard was used for NMR spectroscopic analysis. Excitation and emission wavelengths of the compounds were determined using a Thermo scientific spectrofluorometer (Varioskan Flash model multi-plate reader) instrument. HPLC studies were performed using Agilent technology 1260 Infinity instrument with either an Agilent 1260 Infinity Fluorescence Detector, or a DAD detector (Agilent 1260 Infinity Diode Array) equipment.

5.2 Synthesis of the molecules

The title molecules were synthesized according to the previous methodologies described (Figure 5) [5,13]. Briefly, 2 mmol of 2-bromobenzoic acid derivative (either 2-bromobenzoic acid or 5-hydroxy-2-bromobenzoic acid) dissolved in 7 mmol sodium hydroxide containing 20 mL aqueous solution was mixed with 6 mmol of resorcinol in 10 mL aqueous. The mixture was heated under reflux for 35 min and then it was added 8mL of 20% CuSO4 containing aqueous. After stirring under reflux for another five minutes, the reaction was cooled to room temperature. The precipitate formed was filtered off and washed with acidified water to yield out urolithin A or urolithin B.

HO OH + Br COOH R — NaOH, H_2O → HO R; R: -H, -OH; Urolithin A and B — NaH, MeI / THF → O R_1; R_1: -H, $-OCH_3$; Urolithin A and B methyl ethers

Figure 5. Synthesis of the title molecules

The urolithins A and B obtained were also employed to synthesize their methyl ether derivatives. For this purpose, 1.5 mmol of the title urolithin (either urolithin A or B) was dissolved in 20 mL of THF and the solution added 4 mmol of sodium hydride. Following stirring at room temperature for 5 min, a mixture was added 9 mmol of methyl iodide. At the end of the reaction period (1h), the mixture was poured into 20 mL cold water, followed by the addition of 25 mL ethyl acetate. The organic phase was collected and the remaining aqueous phase was extracted one more time with ethyl acetate and the combined organic phases were removed under reduced pressure to obtain the title methyl ether analogues.

5.3 Preparation of stock and sample solutions of the title molecules

The stock solutions prepared were used to obtain the sample solutions 230, 156, 78, 39, 19.5 ng/mL for the urolithin A and B, whereas 5000, 3750, 2500, 1520, 625 ng/mL solutions for their methyl ether derivatives, all freshly made ready.

5.4 The chromatographic system

The fluorescent properties of the title compounds have been shown by our research group previously. All display large Stokes shifts, around 90 nm. Accordingly, 330nm and 420 nm were respectively used as the maximum excitation and emission wavelengths. In HPLC studies, C/N 5020-01811 (5 µm 4.0 × 150 mm) model Inertsil ODS-3V validation support column was used with the mobile phase composed of A: Water-formic acid (99.9-0.1 v/v) and B: Acetonitrile70% with a flow rate of 0.5mL/min. The column temperature was set to 25ºC and the run times were 11 min.

5.5 Method validation studies

ICH Q2 (R1) guidelines were used for the analytical method validation [27]. Accordingly, specificity, linearity, accuracy, precision, the limit of detection (LOD), and the limit of quantification (LOQ) were the parameters assessed. All measurements were done in triplicates and the calibration curves were obtained through the plotting of concentration vs the peak area both for UV and fluorometric determinations.

Specificity assays were conducted on both standard solutions and blank. Theoretical factors as well as a tailing factor were taken into consideration. Linearity studies were achieved employing the fluorescence intensities of the title molecules. Accordingly, 625-5000 ng/mL range was used for the methyl ethers of urolithin A and B, while the confirmation of the linearity was monitored at the 19.5-230 ng/mL concentration range for both Urolithin A and B. The known (standard weighed) amounts of the title molecules (i.e., 50%, 100%, 150%) were used to test the accuracy of the method by monitoring the closeness of standard reference and observed values. Intraday and inter-day variations in concentration calculations through the relative standard deviations were assessed for precision analysis. LOD values (i.e., the lowest quantity that can be detected but not measured) were found through the equation, $LOD = 3.3 \times Sy/S$, in which Sy represents the standard deviation of the response (Sy) of the curve, and S stands for the slope of the calibration curve. LOQ, the smallest amount that can be measured was calculated with the equation, $LOQ = 10 \times Sy/S$ [28-29].

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