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Identification and quantification of two biologically active polyisoprenylated benzophenones xanthochymol and isoxanthochymol in *Garcinia* species using liquid chromatography–tandem mass spectrometry

Sunil K. Chattopadhyay *, Satyanshu Kumar

Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow 226015, India

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Abstract

A sensitive liquid chromatography/electrospray ionization tandem mass spectrometrical (LC/ESI–MS/MS) method was developed for the identification and quantification of two polyisoprenylated benzophenones xanthochymol and isoxanthochymol in the extracts of the fruit rinds, stem bark, seed pericarps and leaves of *Garcinia indica* and in the fruit rinds of *Garcinia cambogia*. The separation of xanthochymol and isoxanthochymol was achieved on a RP-18 column using the solvent system consisting of a mixture of acetonitrile–water (9:1) and methanol–acetic acid (99.5:0.5) as a mobile phase at a flow rate of 0.4 ml/min. A multiple reaction monitoring (MRM) method was developed for quantification of xanthochymol and isoxanthochymol in the above extracts of *Garcinia* species. On the basis of signal to noise ratio of 3, the limits of detection in MRM mode for xanthochymol and isoxanthochymol were 1.0 ng/ml and 0.5 ng/ml, respectively. The method was validated in terms of linearity, accuracy and precision for 6 days. The method developed was found to be useful for identification and quantification of xanthochymol and isoxanthochymol in the extracts of the fruit rinds, stem bark, seed pericarps and leaves of *G. indica* and in the fruit rinds of *G. cambogia*.

Keywords: Xanthochymol; Isoxanthochymol; Liquid chromatography; Tandem mass spectrometry; Multiple reaction monitoring; *Garcinia* extracts

1. Introduction

Garcinia (family: *Guttiferae*) is a large genus of polygamous trees or shrubs, distributed in tropical Asia, Africa and Polynesia. Its fruit is anthelmintic and cardiotonic. The dried rind of *Garcinia indica* ('Kokum') is used as a garnish for curry and in traditional medicine in India [1]. Xanthochymol and isoxanthochymol (Fig. 1) belong to prenylated benzophenone class of compounds. Pure xanthochymol has been isolated from *Garcinia xanthochymus, Garcinia mannii, Garcinia stauditii, Rheedia madrunno, Garcinia subelliptica* and *Garcinia pyrifera*. Isoxanthochymol has also been isolated from *G. xanthochymus, G. subelliptica* and *G. pyrifera* [2]. Recent studies have demonstrated that polyprenyl benzophenones xanthochymol and guttiferone E isolated from the ethyl acetate soluble extracts of fruits of *G. pyrifera* exhibited significant inhibitory activity on the disassembly of microtubules into tubulin [2]. It has also been reported that xanthochymol exhibited significant growth suppression in human leukemia cell line because of apoptosis mediated by caspase-3 [3]. Moreover, xanthochymol showed strong antimethicillin resistant *Staphylococcus aureus* activity which almost equals to vancomycin [4].

As a part of our ongoing research program for isolation of paclitaxel (taxol) mimics from higher plants, we have taken up a systematic investigation on the chemical constituents of the fruits of *G. indica* and have isolated xanthochymol and isoxanthochymol from its extract (Fig. 1). There has not been any report in the literature for a sensitive and reliable method for the analysis of the above two important molecules. Recently, we have developed an HPLC method for the quantification of xanthochymol and isoxanthochymol in the fruit rinds of *Garcinia* species with the lowest limit of quantification (LLOQ) as 20 μg/mL and 5 μg/mL for xanthochymol and isoxanthochymol, respectively

* Corresponding author. Tel.: +91 522 2717529x212; fax: +91 522 2342666.
E-mail address: chattsk@yahoo.com (S.K. Chattopadhyay).

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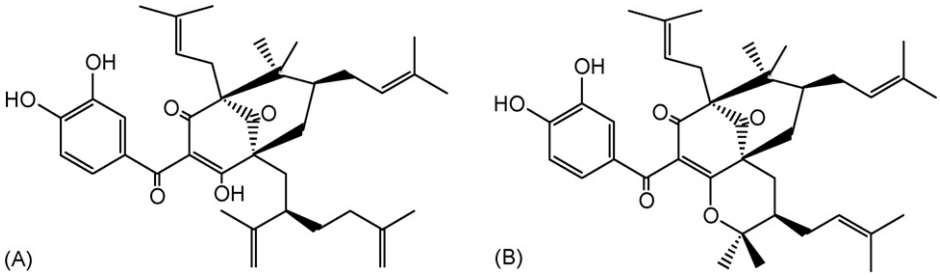


Fig. 1. Structures of xanthochymol (A) and isoxanthochymol (B).

[5]. However, the sensitivity of the HPLC was found to be inadequate for quantifying xanthochymol and isoxanthochymol in extracts of seeds, and stem bark of *G. indica* and fruit rinds of *G. cambogia* as the contents of xanthochymol and isoxanthochymol were below the lowest limit of quantification (LLOQ). Since we would also be screening different parts of *Garcinia* species for the presence of the above two molecules, it was felt necessary to develop a rapid and sensitive LC-ESI/MS/MS method for identification and quantification of xanthochymol and isoxanthochymol in the extracts of the above plants. In this paper, we are going to report a LC-ESI/MS/MS method which can detect, identify and also quantify xanthochymol and isoxanthochymol at a ppb level in the extracts of fruit rinds, stem bark, seed pericarps and leaves of *G. indica* and in the fruit rinds of *Garcinia cambogia*. The method developed would be useful for identification and quantification of the above two molecules xanthochymol and isoxanthochymol in the extracts of other plant species.

2. Experimental

2.1. Chemicals and materials

The fruit rinds of *G. indica* were collected from Bangalore, India. Also, the fruit rinds, stem barks, seeds and leaves of *G. indica* were collected from Dapoli in the province of Maharashtra, India. The fruit rinds of *G. cambogia* were collected from Kerala, India. The plant materials were dried under shade at room temperature 25 ± 2 °C and powdered in an electric grinder. The powdered plant materials were used for the extraction of xanthochymol and isoxanthochymol. All solvents used were of HPLC grade (Merck, Mumbai, India). Ultra pure distilled water with resistivity greater than 18 MΩ was used. For samples and solvent filtration, 0.45 μm membrane filters (Millipore, Germany) were used, and solvents were degassed prior to use.

2.2. Extraction of plant material

The methanol extract of fruit rinds of the *G. indica* was prepared as follows. Fruit rinds were air-dried in the shade at room temperature, and the dried materials were powdered. Then, 100 g of the powder was extracted with methanol (500 ml) for 48 h at room temperature. The crude preparation was filtered and concentrated under reduced pressure to provide a crude extract (42 g) which was used for analysis. Xanthochymol and isoxanthochymol were isolated from a methanolic extract of the fruit rinds of *G. indica* following a standard protocol [6]. The purity (98%) and structures of xanthochymol and isoxanthochymol were confirmed by matching their reported physiochemical (mp, optical rotations) and spectral data (NMR, Mass, UV, IR) [6]. These two isolated and characterized molecules were used as standard for HPLC and ESI–MS analyses as standard xanthochymol and isoxanthochymol are not commercially available.

n-BuOH extract was prepared from the methanolic extract of *G. indica* by treating the methanol extract with water and partitioning it with hexane, chloroform, ethyl acetate and finally with *n*-BuOH successively. *n*-BuOH fraction and aqueous layer left after *n*-BuOH extraction were concentrated under reduced pressure to give the *n*-BuOH and aqueous extract of *G. indica*, respectively. Aqueous extract of *G. cambogia* was prepared from the methanolic extract of *G. cambogia* by treating the methanol extract with water and partitioning it with hexane, chloroform, ethyl acetate successively. The aqueous layer left after ethyl acetate extraction was concentrated under reduced pressure to give the aqueous extract of *G. cambogia.*

2.3. Chromatographic conditions

A Perkin-Elmer Series 200 HPLC system (Perkin-Elmer, USA) with an auto injector was used to deliver the mobile phase [acetonitrile: H_2O (90: 10) (30%) (solvent A) and 0.5% AcOH in MeOH (70%) (pH 2.4) (solvent B)] at a flow rate of 0.4 ml/min in a isocratic elution mode at 23 °C. Chromatographic separation were achieved on a Brownlee RP-18 column (100 mm × 2.1 mm I.D., 5 μm particle size) preceded with a guard column packed with the same material. The samples (10 μl) were injected into the LC-MS/MS system.

2.4. Mass spectrometric conditions

The API-3000 LC-MS/MS (Applied Biosystems/MDS SCIEX, Toronto, Canada) mass spectrometer was operated using a standard ESI source coupled with a LC separation system. Analyst software (Version 1.4, Applied Biosystems/MDS SCIEX, Toronto, Canada) was used for the control of equipment, acquisition and data analysis. MS parameters were optimized by constant infusion of a known concentration of xanthochymol and isoxanthochymol. MS scan was performed in both positive and negative mode and declustering potential was optimized. For the product ion spectrum

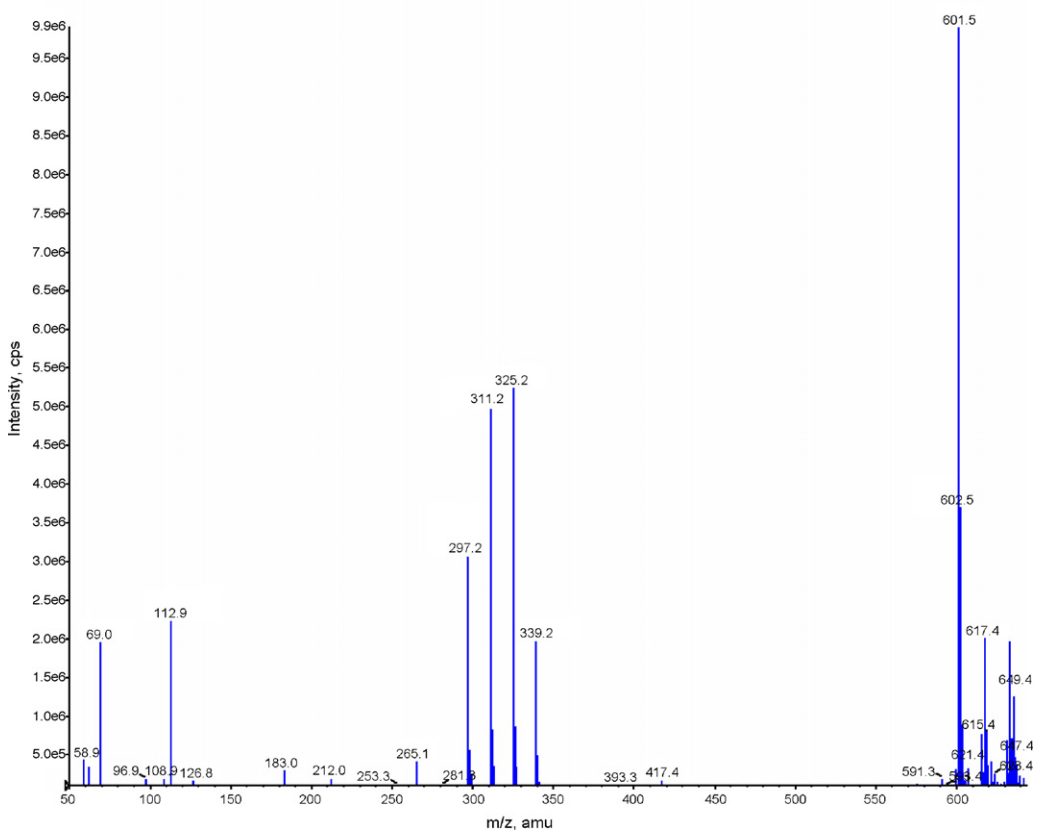


Fig. 2. Q1 MS spectrum of xanthochymol in negative ion mode.

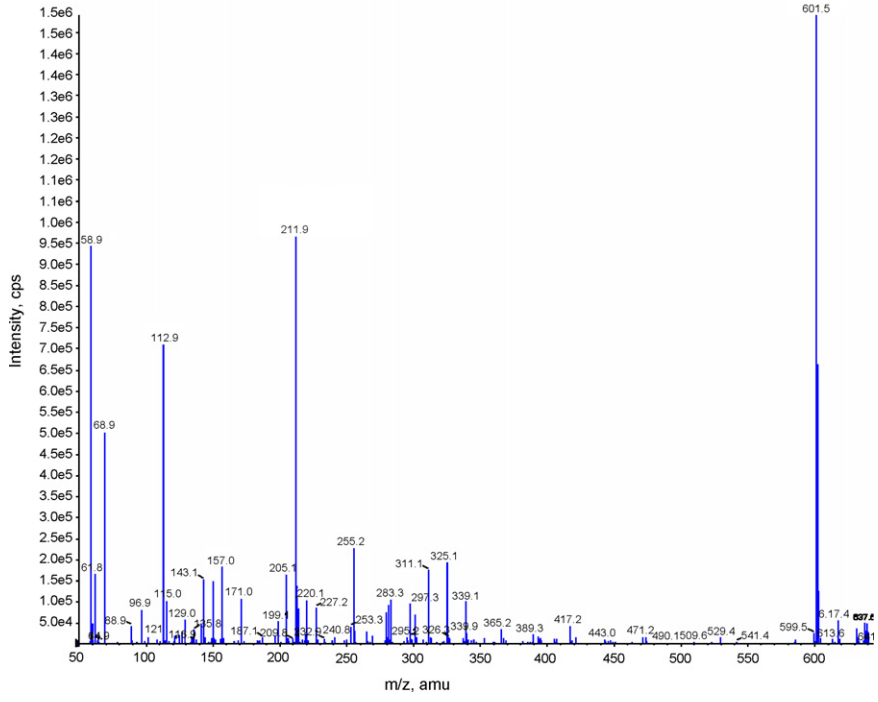


Fig. 3. Q1 MS spectrum of Isoxanthochymol in negative ion mode.

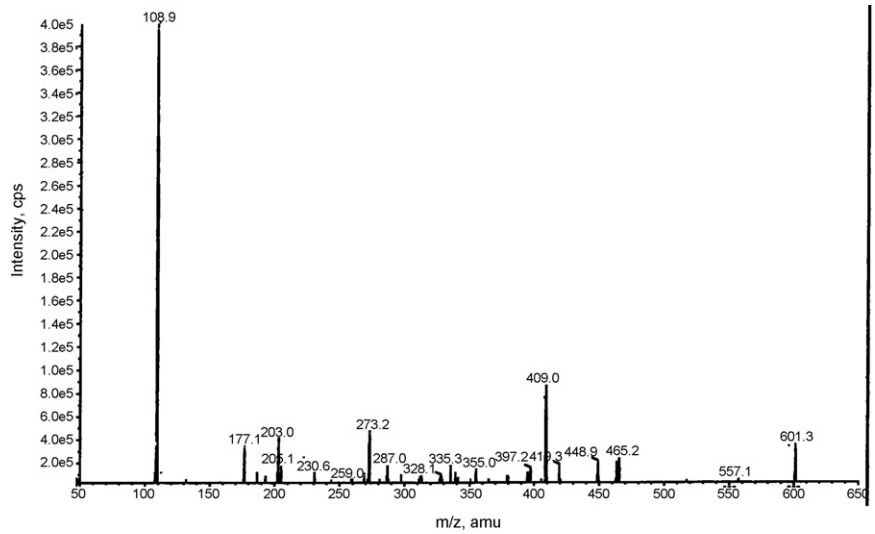


Fig. 4. Product ion spectra of xanthochymol in negative ion mode.

(MS–MS) optimization, the optimized declustering potential was used with nitrogen as the collision gas to obtain prominent product ions.

2.5. Standards and working solutions

Xanthochymol (1 mg/ml) and isoxanthochymol (1 mg/ml) were prepared in HPLC grade methanol. Working stock solutions of xanthochymol (1–150 ng/ml) and isoxanthochymol (0.5–50 ng/ml) were prepared by further dilution of respective stock solution in methanol. Stock solutions of different extracts of *G. indica* and *G. cambogia* were prepared by dissolving 1 mg of the extract in 1 ml methanol and filtered through a 0.45 μm membrane filter. Stock solutions of the extract were further diluted with methanol to get a final concentration of 1 μg/ml. Quality control samples of xanthochymol [at low (10 ng/ml), medium (25 ng/ml) and high (100 ng/ml)]and isoxanthochymol [at low (10 ng/ml), medium (25 ng/ml) and high (50 ng/ml)] were prepared in triplicate each day.

2.6. Method validation

The validation of LC-MS/MS method included within- and between run accuracy and precision determination of quality control samples in triplicate for six different days. MRM in negative mode was utilized for quantitation.

2.6.1. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) of the method for xanthochymol and isoxanthochymol was the lowest quantity in sample corresponding to three times the baseline noise(S/N > 3) and the limit of quantification (LOQ), which is defined as the lowest concentration that can be determined with acceptable accuracy and precision at a S/N ratio>10.

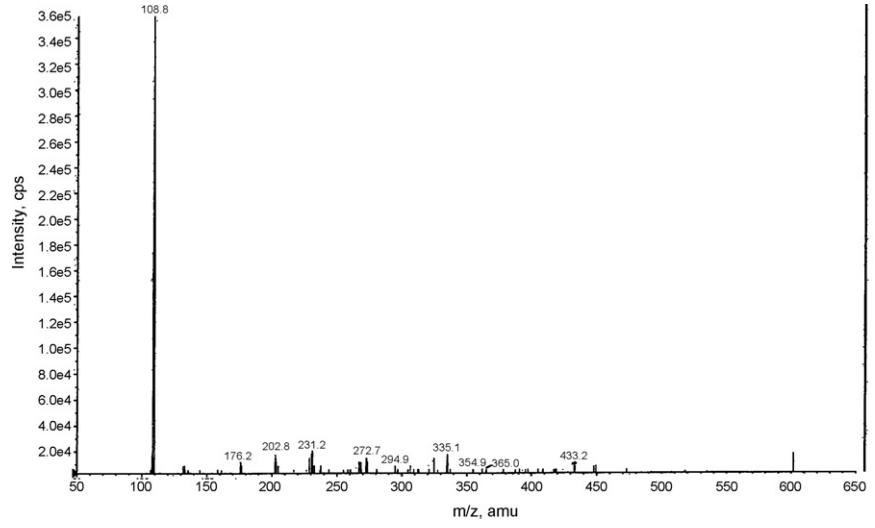


Fig. 5. Product ion spectra of isoxanthochymol in negative ion mode.

2.6.2. Specificity, accuracy and precision

The specificity of the method was defined as non-interference in the regions of interest, for quantifications of xanthochymol and isoxanthochymol. Six replicates of standard extract (blank samples) were analyzed to ensure that no interference with the mass transitions chosen for xanthochymol and isoxanthochymol took place. Method reproducibility was evaluated by six injections of standard solutions and six replicates analysis of sample solutions, respectively. The precision of the method expressed as a relative standard deviation (R.S.D.) was calculated by six replicate injections of three different concentrations (low, medium and high) of standard xanthochymol and isoxanthochymol. The intra-day and inter-day R.S.D.s (%) of the developed LC-MS/MS method were obtained in the range of 2.94–5.76 and 6.23–9.82 for xanthochymol and 1.42–4.61 and 3.01–4.97 for isoxanthochymol, respectively (Table 2)

3. Results and discussions

3.1. Optimization of LC and MS conditions

LC conditions were optimized to the conditions described in experimental section using the mobile phase [acetonitrile: H_2O (90: 10) (30%) (solvent A) and 0.5% AcOH in MeOH (70%) (solvent B)] at a flow rate of 0.4 ml/min in an isocratic elution mode. Out of different columns tried, best results were obtained on Brownlee RP-18 column (100 mm × 2.1 mm I.D., 5 μm particle size) preceded with a guard column packed with the same material. The mass spectrometric behaviors of the two compounds were investigated in both positive and negative ion modes. In positive ion mode, no abundant protonated $[M+H]^+$ ion was observed both for xanthochymol and isoxanthochymol. However, in negative ion mode, both the molecules showed abundant $[M-H]^-$ ion. Q1 MS scan for xanthochymol and isoxanthochymol in negative ion modes are shown (Figs. 2 and 3).

3.2. Optimization of MRM conditions

Standard solutions of xanthochymol (4 μg/ml) and isoxanthochymol (2 μg/ml) were infused using a Hamilton syringe pump to the mass spectrometer to optimize the sensitivity for the $[M-H]^-$ ion (*m/z* 601.3) for xanthochymol and (*m/z* 601.6) for isoxanthochymol. Purified air was used as a nebulising gas and nitrogen generated with a nitrogen generator (Peak scientific, model NM 20 Z, USA) as turbo, curtain and collision gas. Collision energy (CE) and CAD gas (nitrogen) were optimized for yields of fragments in Collision Activated Dissociation (CAD) experiments. The MS/MS spectra of the $[M-H]^-$ ions of xanthochymol showed three fragments at *m/z* 108.9, 273 and 409 and for isoxanthochymol at *m/z* 108.9,202.6 and 433.2. The product ion spectra of xanthochymol and isoxanthochymol in negative mode are given in Figs. 4 and 5. The proposed fragmentation pathways for xanthochymol and isoxanthochymol are given Fig. 6. The major fragment (*m/z* 108.9) is common in the both xanthochymol and isoxanthochymol and its intensity is very high. Due to the high intensity of the fragment

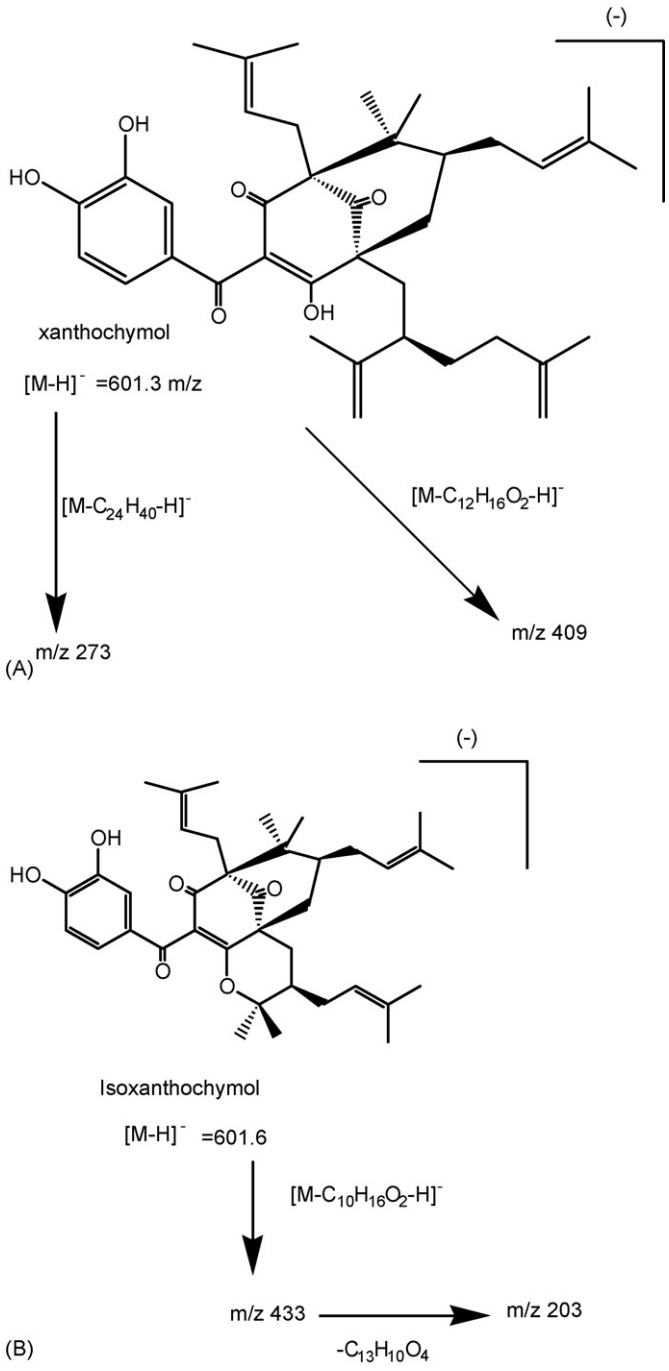


Fig. 6. (A) Proposed fragmentation pathway for xanthochymol; (B) proposed fragmentation pathway for isoxanthochymol.

(*m/z* 108.9), it was not used for developing MRM method for quantification as it was disturbing the base line in MRM chromatogram. The MRM method for quantification was developed using two other transitions (*m/z* 601.3 → 273; *m/z* 601.3 → 409) for xanthochymol and (*m/z* 601.6 → 202.6; *m/z* 601.6 → 433.2) for isoxanthochymol. Xanthochymol and isoxanthochymol are stereo isomeric molecules i.e., they have the same molecular weight (*m/z* 602). Also, they have almost the same fragmentation pattern. But they have different retention times on HPLC (1.09 min for isoxanthochymol and 1.78 min for xanthochymol).

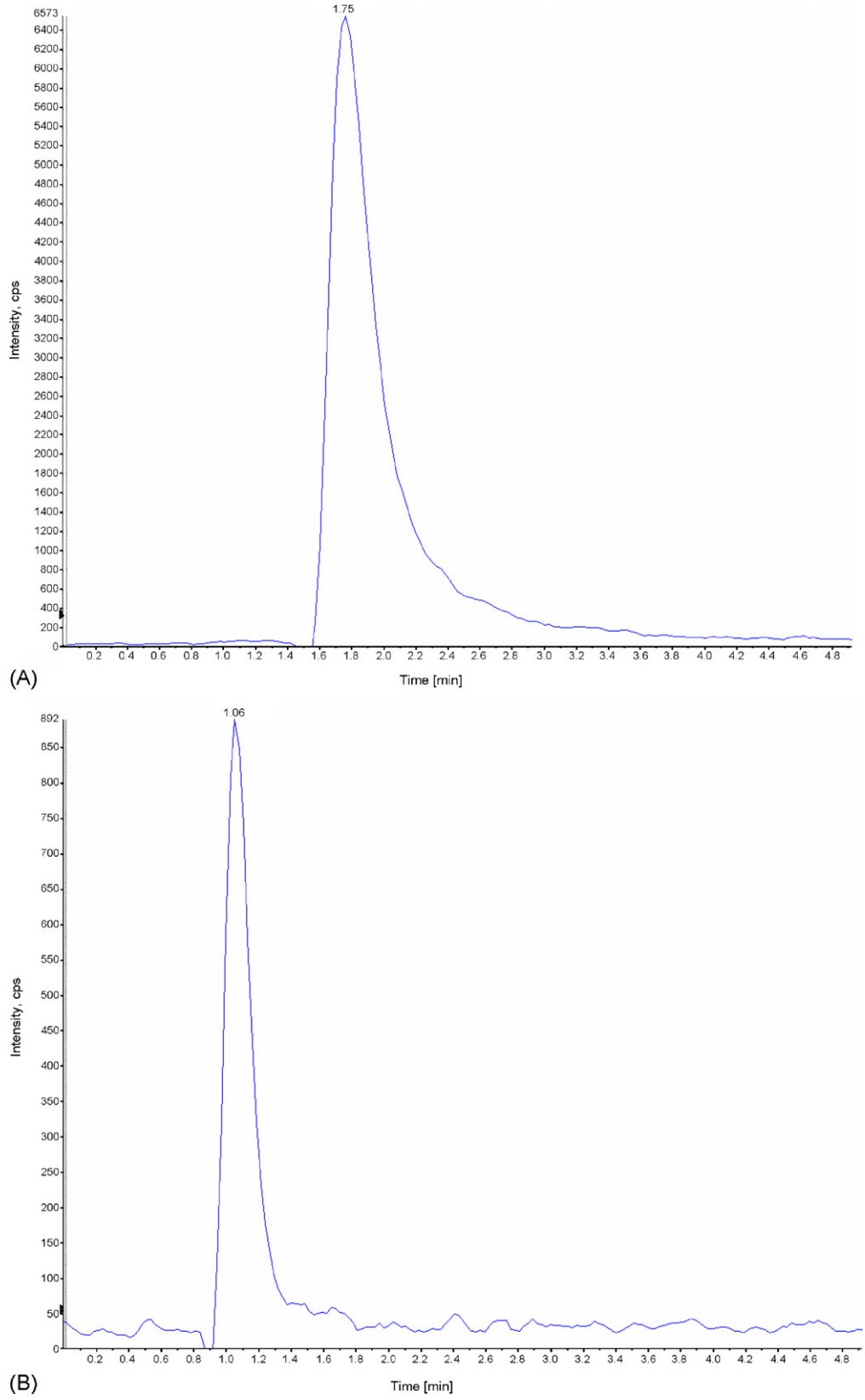
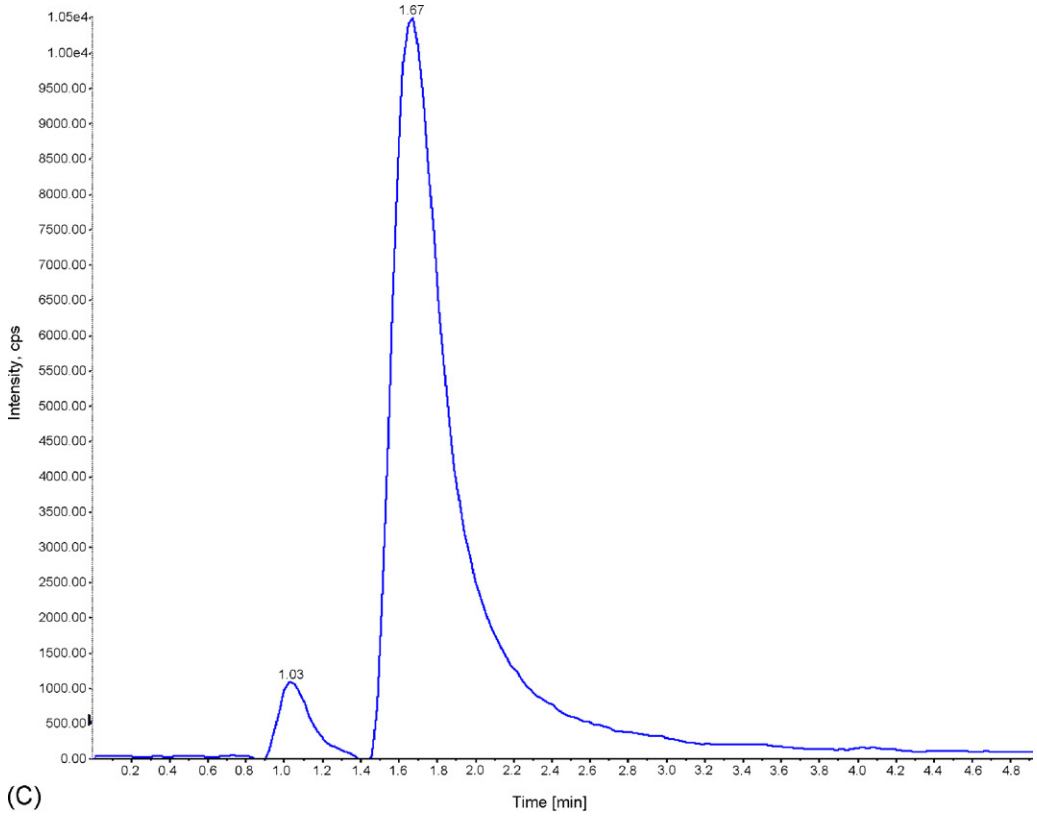
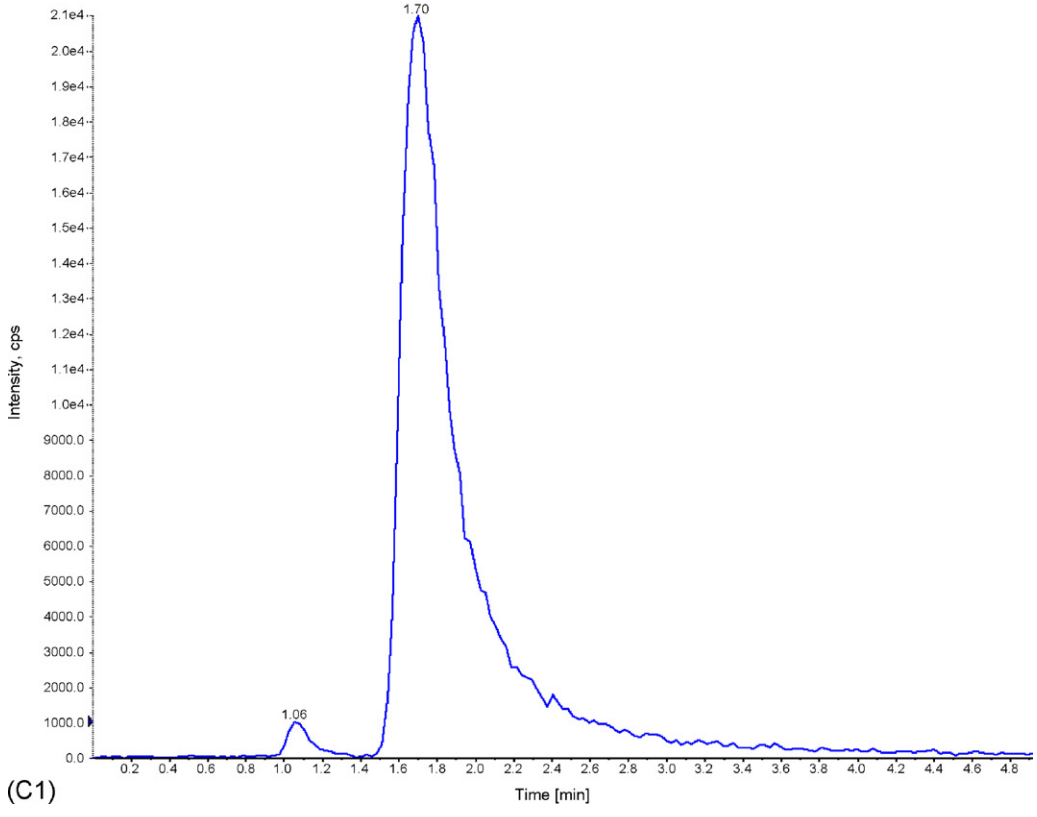


Fig. 7. Representative MRM chromatograms of (A) Xanthochymol standard; (B) isoxanthochymol standard; (C) *Garcinia indica* fruit rind (Bangalore) MeOH extract; (C1) sample C spiked with xanthochymol and isoxanthochymol; (D) *Garcinia indica* fruit rind (Dapoli) MeOH extract; (D1) sample D spiked with xanthochymol and isoxanthochymol; (E) *G. indica* stem bark (Dapoli) MeOH extract; (E1) sample E spiked with xanthochymol and isoxanthochymol; (F) *G. indica* seed pericarp (Dapoli) MeOH extract; (F1)) sample F spiked with xanthochymol and isoxanthochymol; (G) *G. indica* leaves (Dapoli) MeOH extract; (G1) sample G spiked with xanthochymol and isoxanthochymol; (H) *G. indica* fruit rind (Bangalore) aqueous extract; (H1) sample H spiked with xanthochymol and isoxanthochymol; (I) *G. cambogia* fruit rind (Kerala) MeOH extract; (I1) sample I spiked with xanthochymol and isoxanthochymol.





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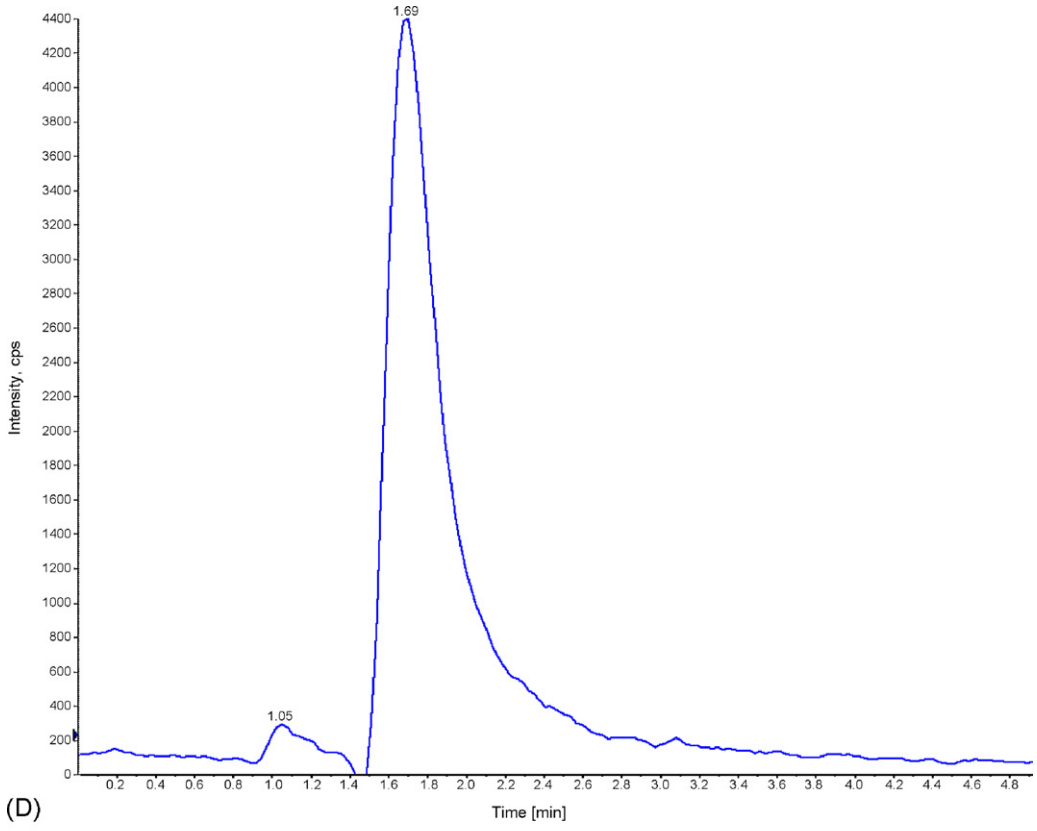

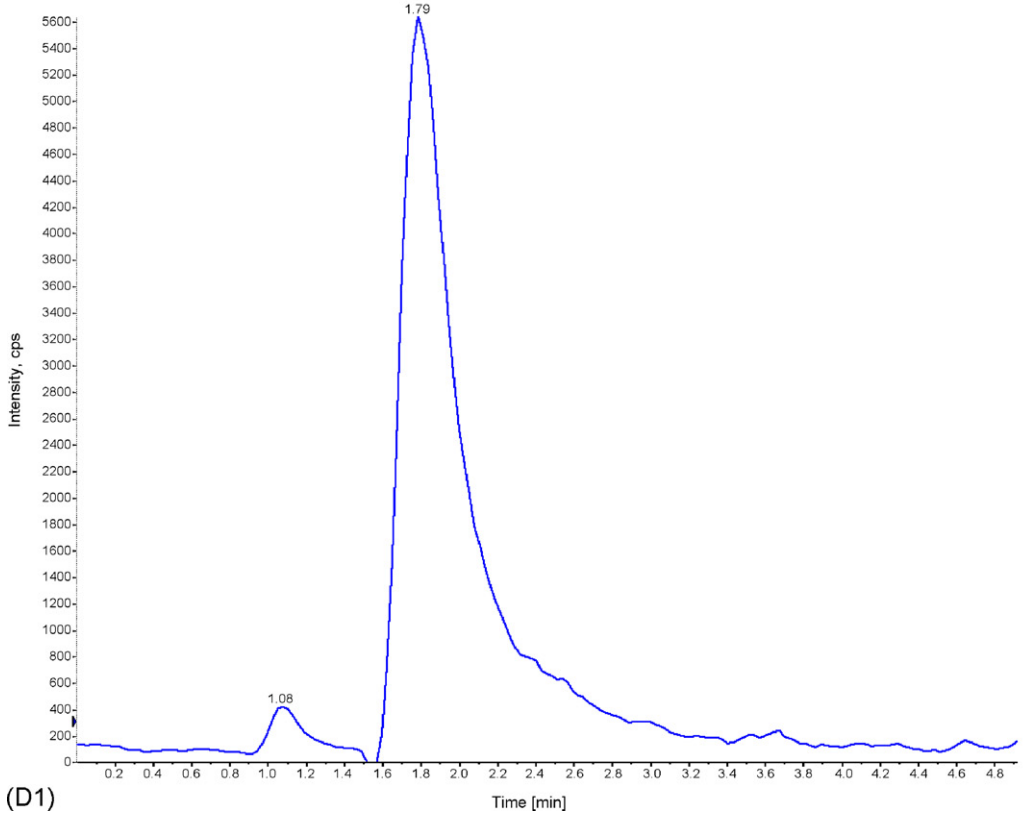

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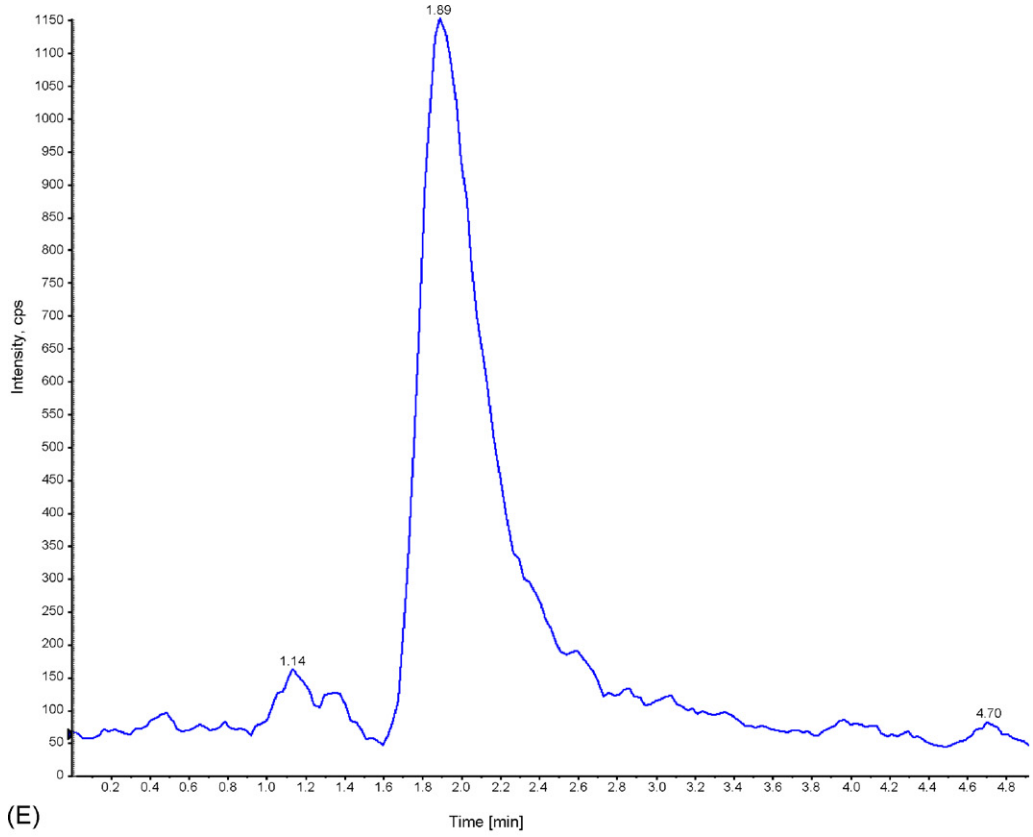

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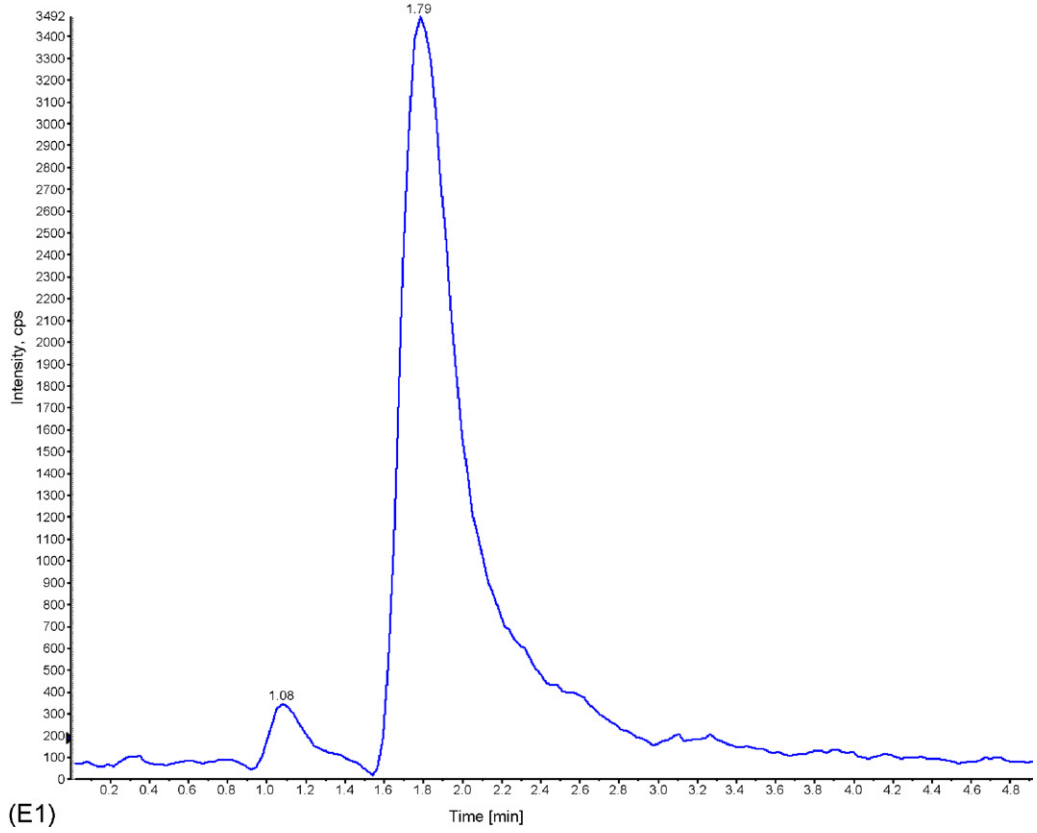

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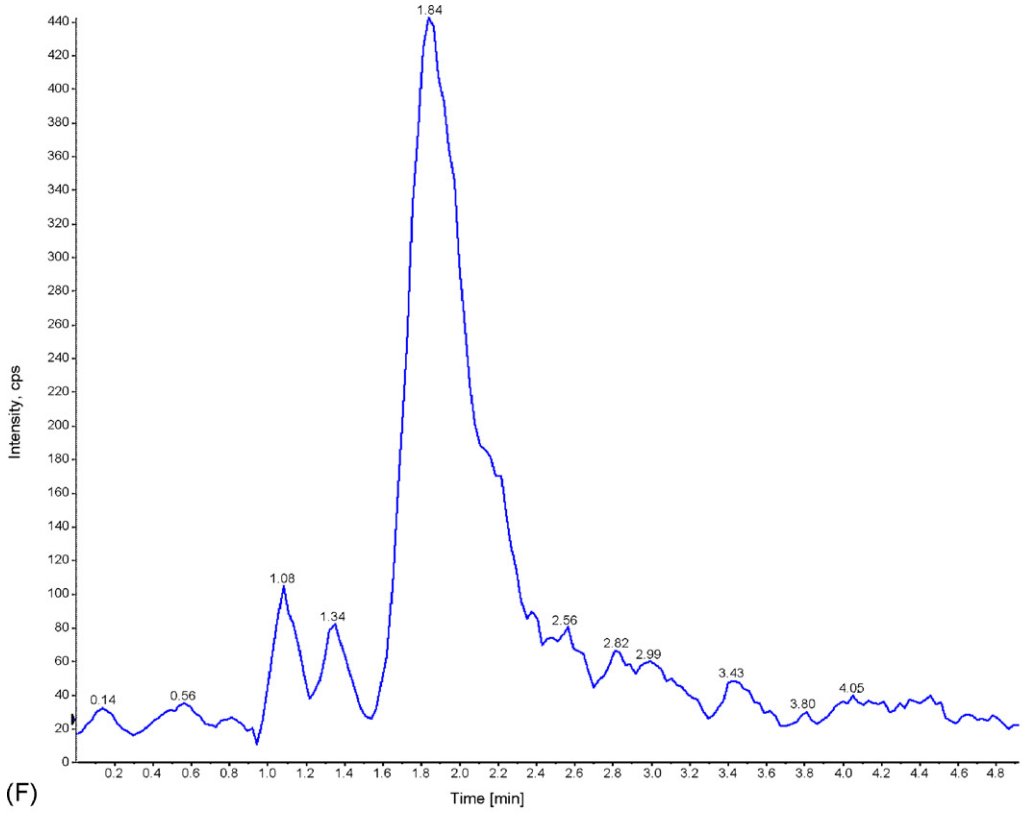

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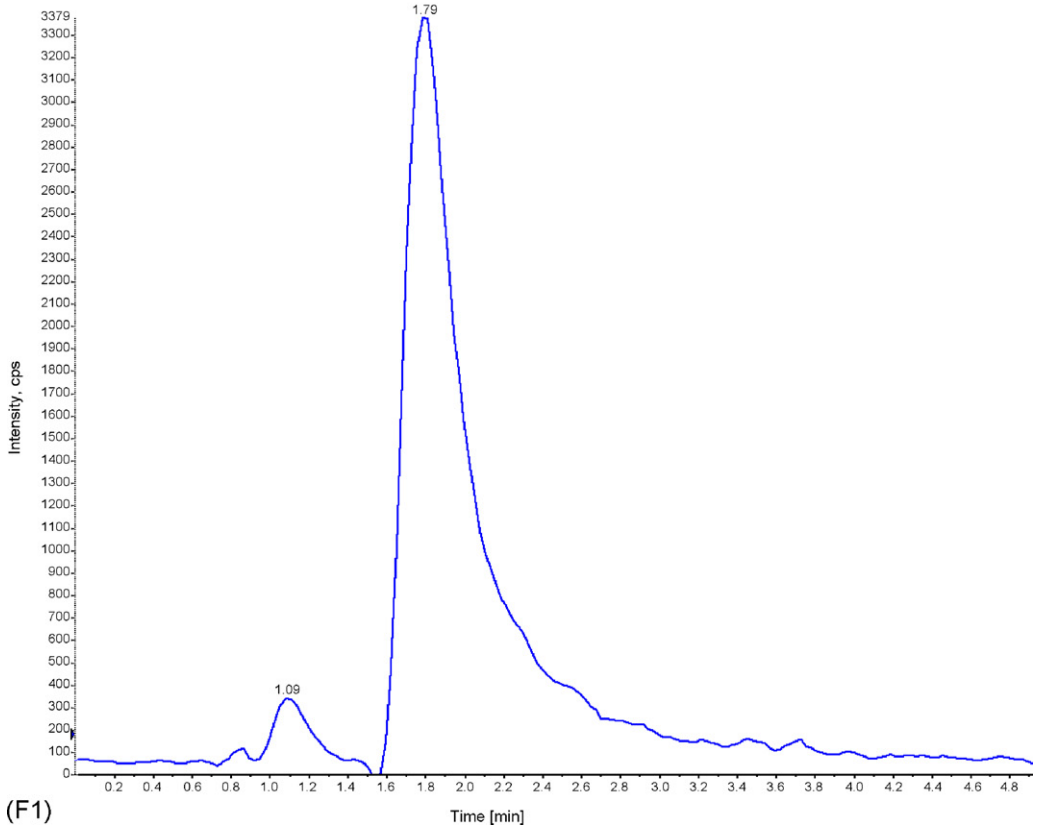

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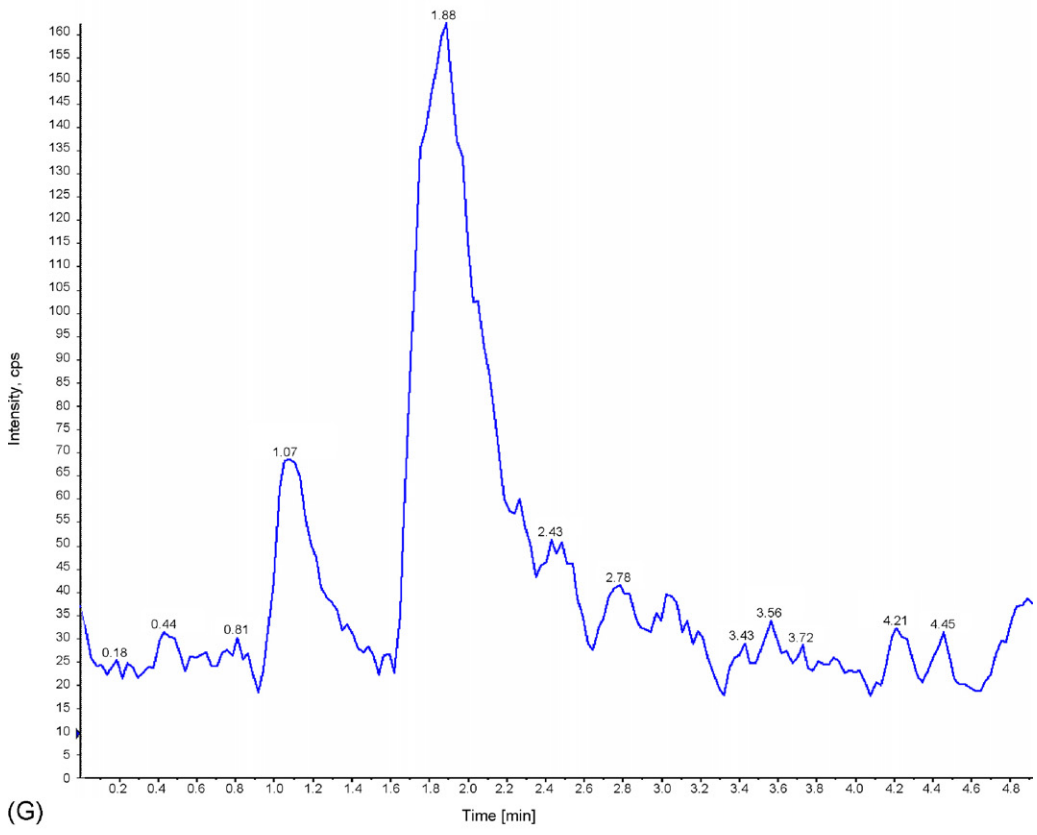

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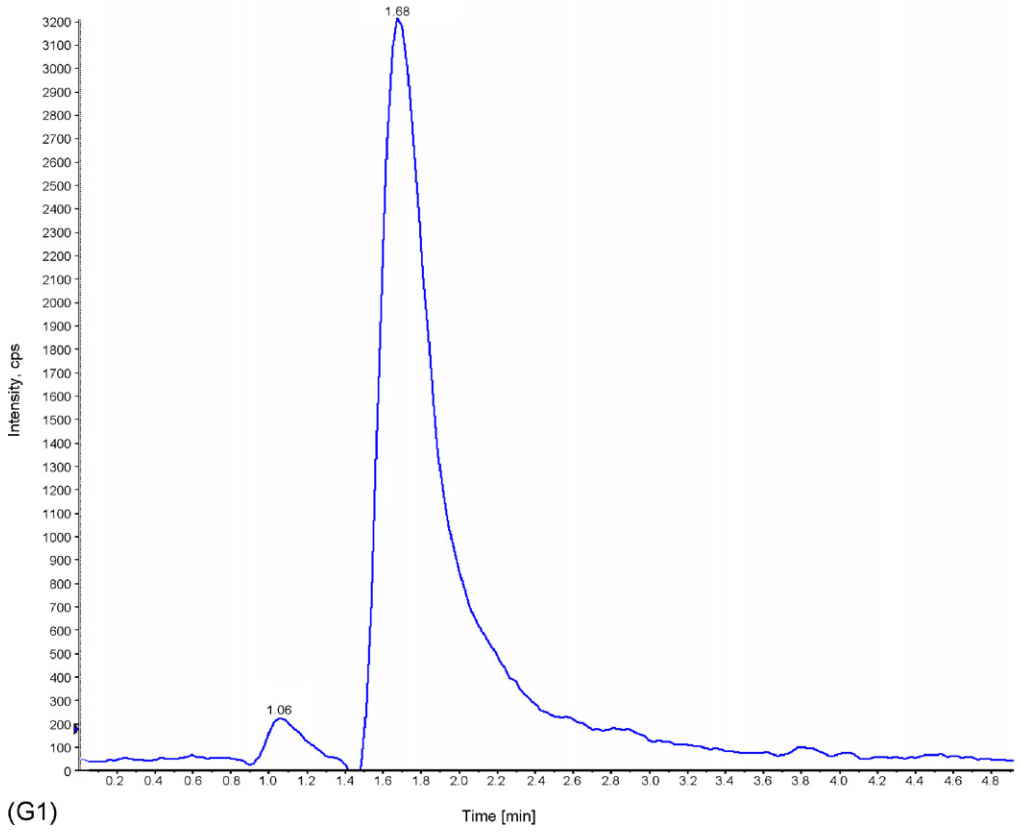

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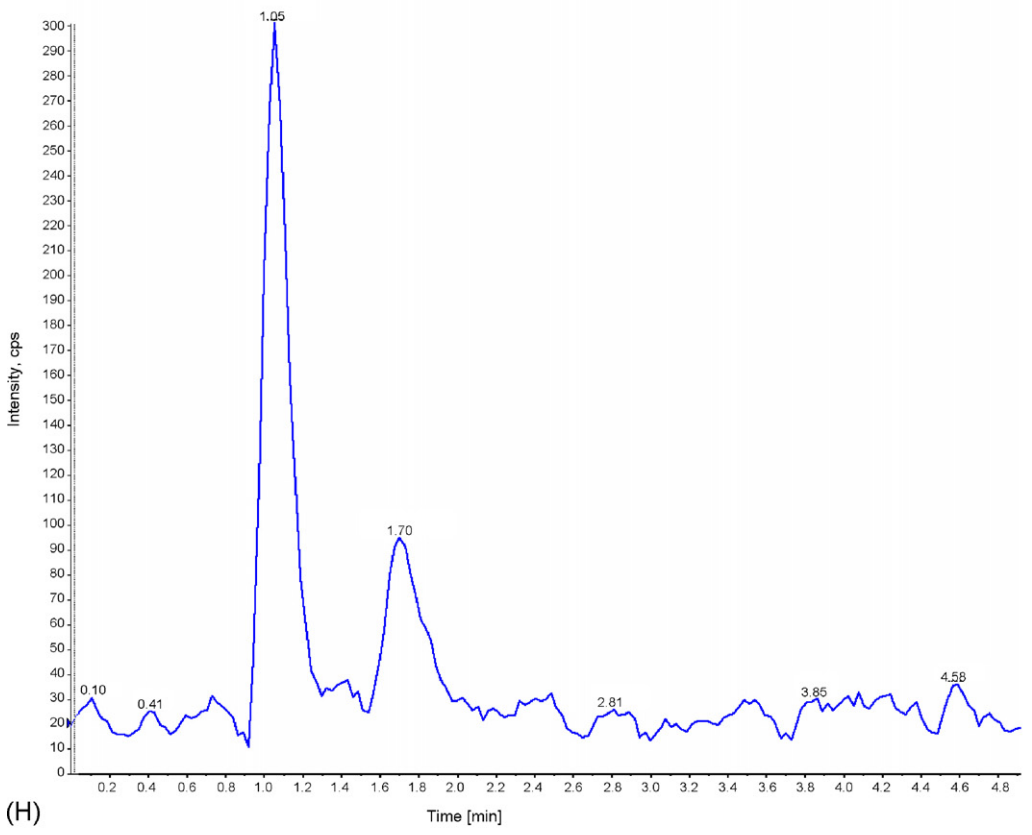

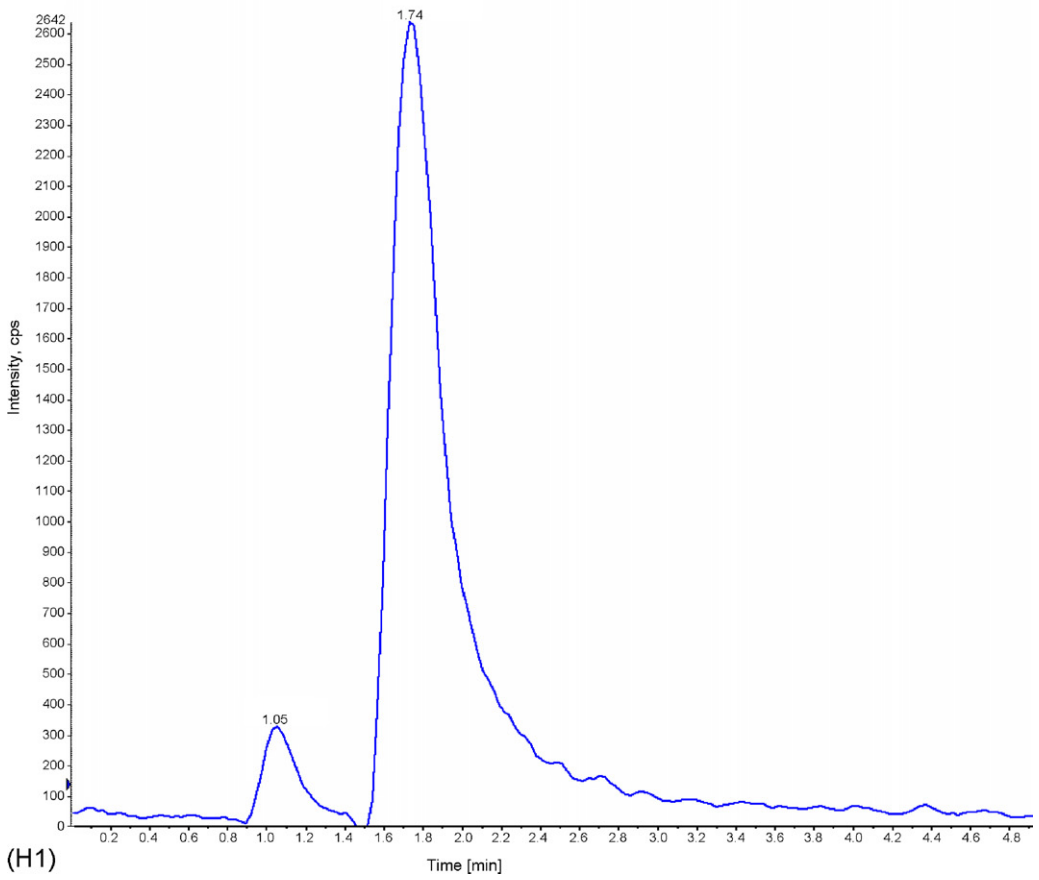

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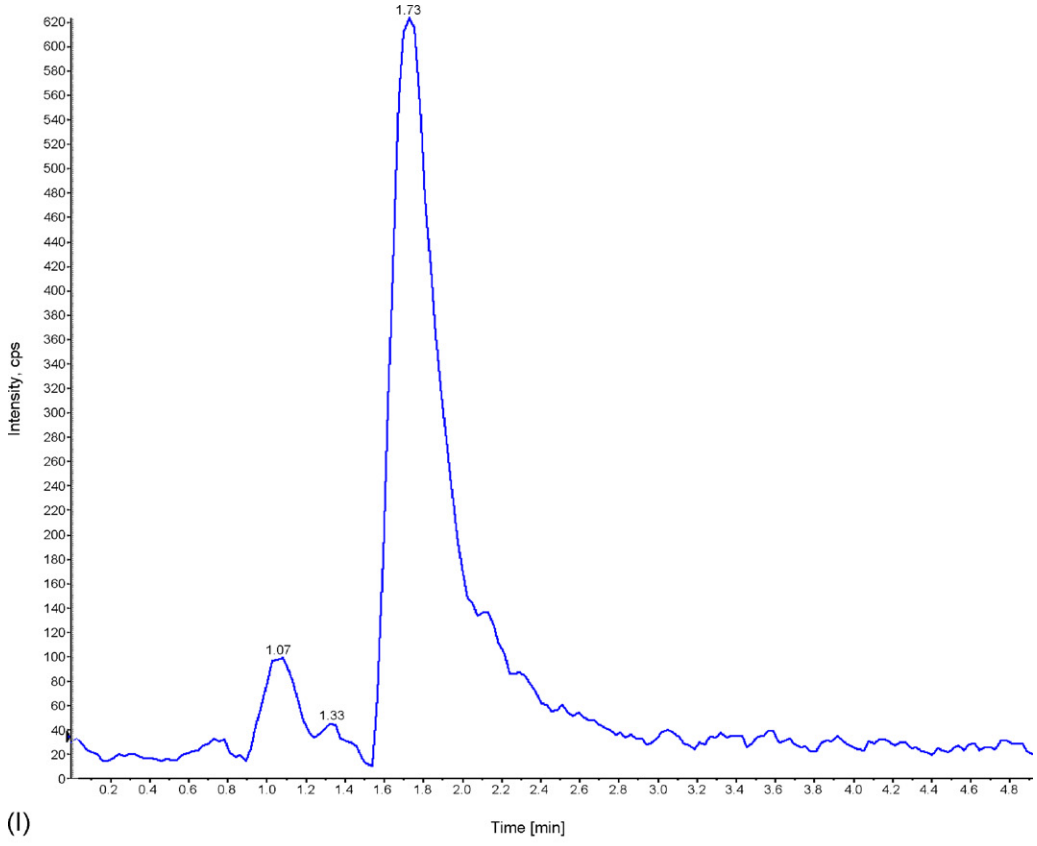

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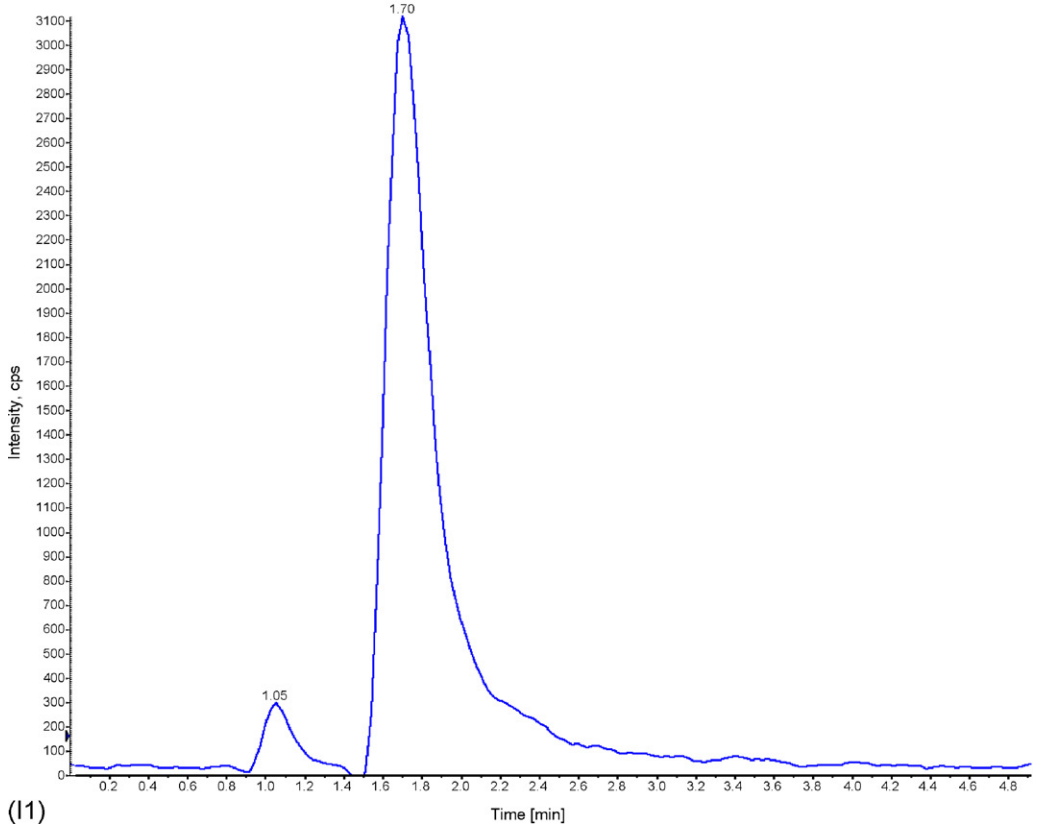

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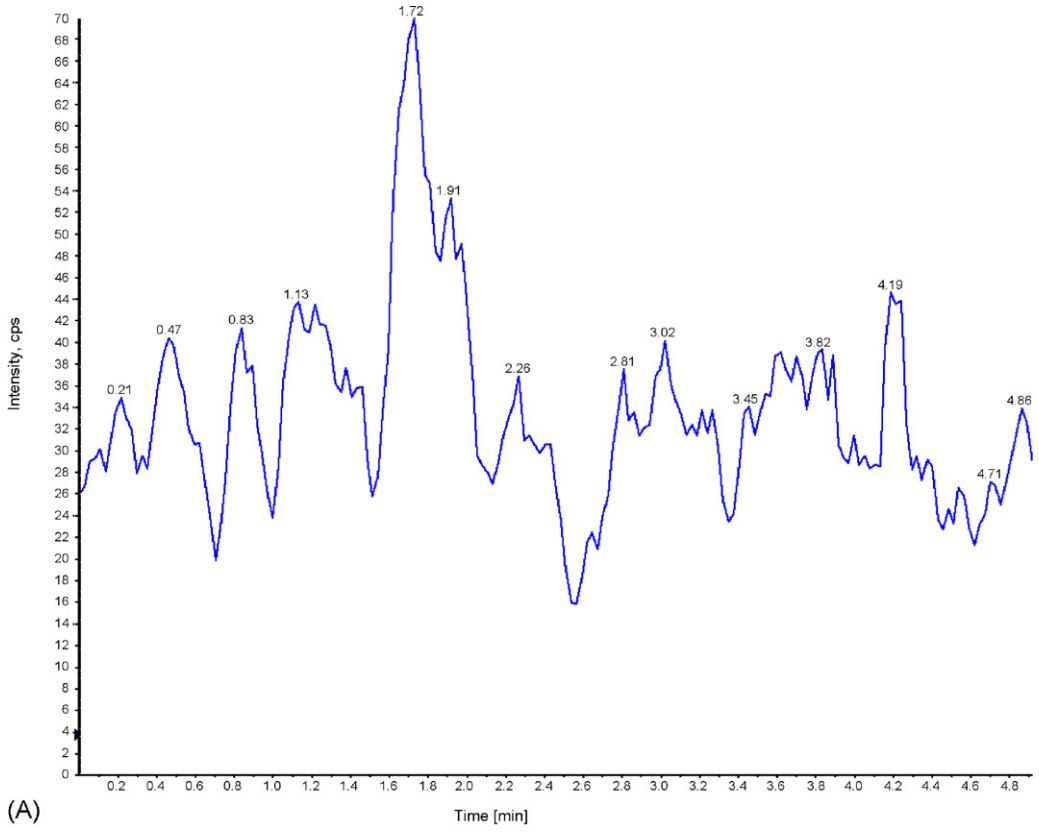


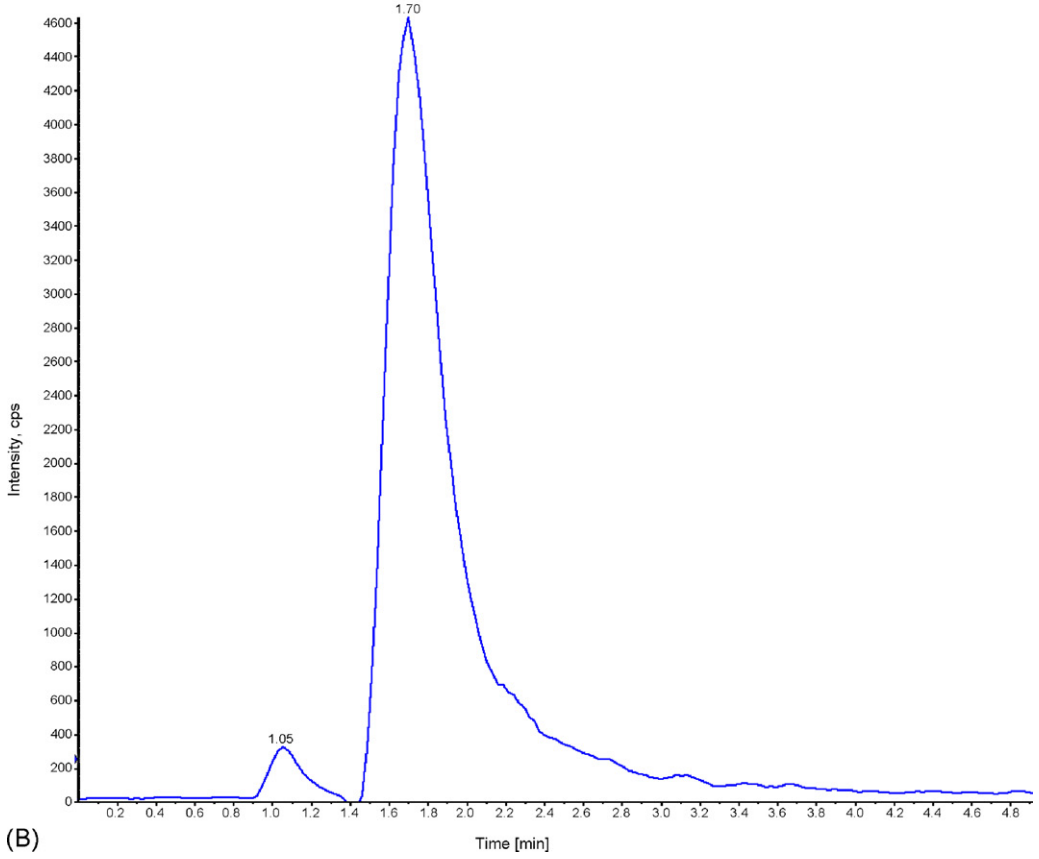
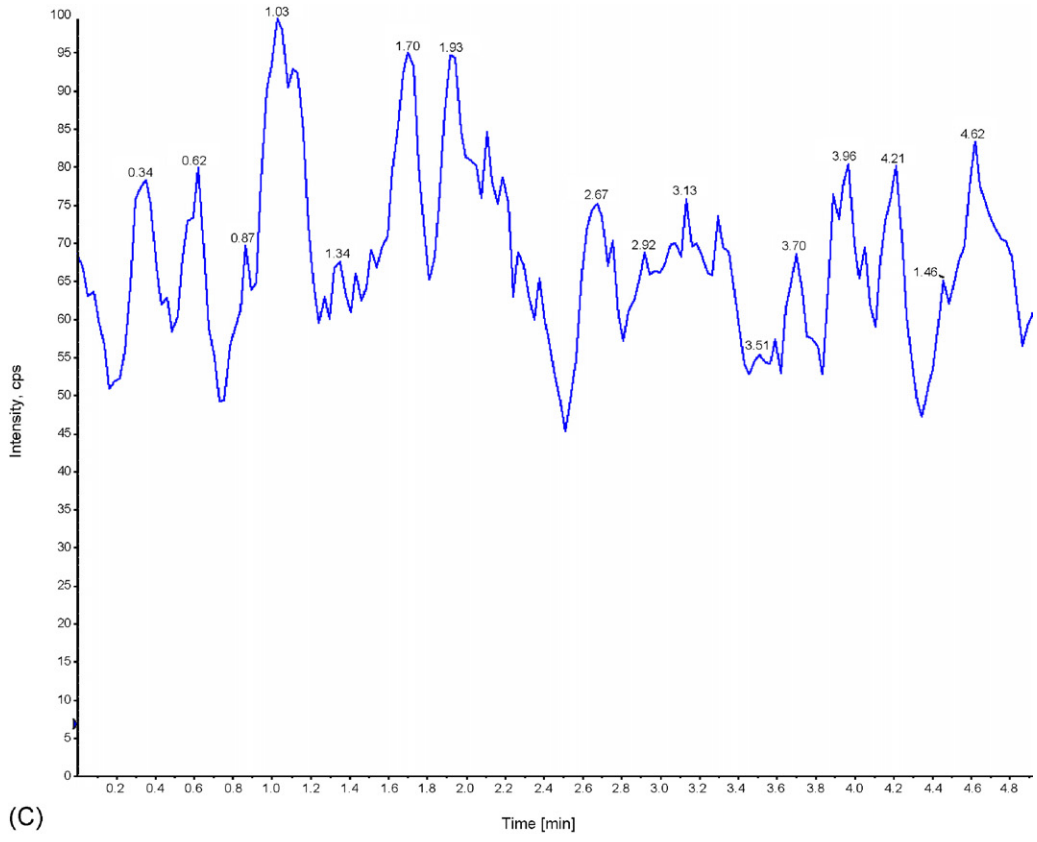


Fig. 8. Representative MRM chromatograms of (A) standard matrix (G.I. fruit rinds *n*-BuOH extract); (B) sample A spiked with xanthochymol and isoxanthochymol; (C) standard matrix (G.C. fruit rinds aqueous extract); (D) sample C spiked with xanthochymol and isoxanthochymol.



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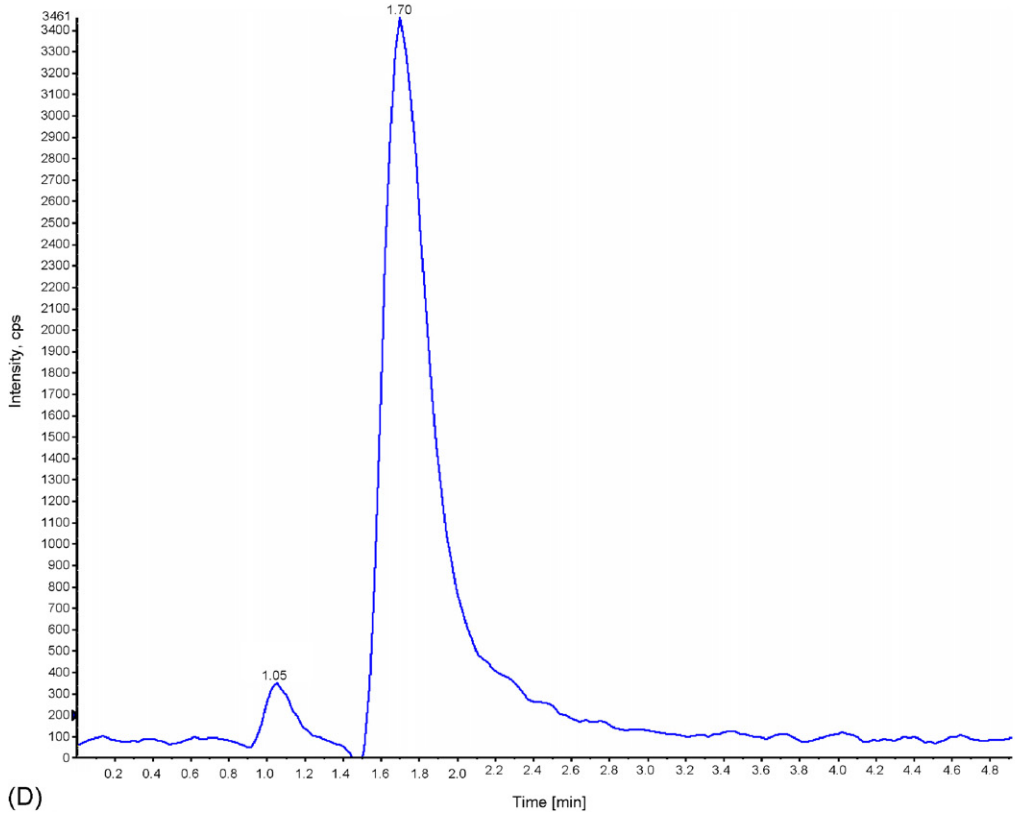

(D)

Fig. 8. (*Continued*).

Table 1
Response characteristics of xanthochymol and isoxanthochymol using LC-MS/MS

Compounds	Retention time (min)		Equation of calibration curve[a]	R^2	Linear range	Detection limit[b]
	Mean	R.S.D. (%)				
Xanthochymol	1.78	0.32	$y = 2730x + 3730$	0.9973	5–125	1
Isoxanthochymol	1.07	0.46	$y = 462x + 416$	0.9978	4–50	0.5

[a] In the calibration equation, x represents concentration of the analyte (ng/ml) and y represents the peak area (cps × s).
[b] The limits of detection were estimated based on the signal/noise = 3.

The fragment (m/z 433.2) is found only in isoxanthochymol and thus it can serve as a qualifier ion for identifying isoxanthochymol from xanthochymol.

The ion spray and orifice (declustering) voltages were optimized to 4000 and −110 V for xanthochymol and 4000 and −69.87 V for isoxanthochymol. The turbo gas flow rate was 7 l/min and it was heated to 500 °C. Analyses were carried out in the negative mode using multiple reaction monitoring (MRM) with the following two reactions: m/z 601.3 → 273; m/z 601.3 → 409 for xanthochymol and m/z 601.6 → 202.6; m/z 601.3 → 433.2 for isoxanthochymol. The dwell time for each reaction was 400 ms. The collision energy was set to −50 eV for xanthochymol and −60 eV for isoxanthochymol. The collision cell exit potential was −5.30 V for xanthochymol and −10 V for isoxanthochymol.

3.3. Method validation

The reproducibility of the retention time of xanthochymol and isoxanthochymol under optimum LC-MS/MS conditions was investigated by doing repeated injections ($n = 6$) of a mixture of the standards at a concentrations of 12.5 ng/ml. The relative standard deviations (R.S.D.s) of retention times (min) for xanthochymol and isoxanthochymol were 0.32% and 0.46%, respectively (Table 1). The good reproducibility in retention time indicated that this method is accurate, robust and would probably be reliable for screening xanthochymol and isoxanthochymol in plant samples. Based on a signal to noise ratio of 3, the limits of detection in MRM mode were 1.0 ng/ml and 0.5 ng/ml for xanthochymol and isoxanthochymol, respectively. The linear equation between the concentration of the standard injected and the peak area can be expressed as $y = mx + c$, where y is the peak area and x is concentration of the standard and m and c are constants. A good linearity of the method was found over the investigated calibration range of 5–125 ng/ml and 4–50 ng/ml for xanthochymol and isoxanthochymol, respectively, based on 6-level calibration curves. Two independent injections were carried out for every calibration point. Typically, the coefficients of correlation (r^2) of this method were above 0.997 for xanthochymol and isoxanthochymol, respectively. The quantitative data obtained from the analysis of xanthochymol and isoxanthochymol by LC-MS/MS were summarized in Tables 1 and 2.

Table 2
Accuracy and precision (R.S.D.) of the method at three different concentrations

Analyte	Concentration (ng/ml)	R.S.D. (%)	
		Intra-day	Inter-day
Xanthochymol	10	5.76	6.99
	25	2.94	6.23
	100	3.17	9.82
Isoxanthochymol	10	4.61	4.97
	25	1.91	7.49
	50	1.42	3.01

3.4. Analysis of xanthochymol and isoxanthochymol in Garcinia extracts

Under optimum LC-MS/MS conditions, the presence of xanthochymol and isoxanthochymol in the extracts of *Garcinia* samples was successfully identified and quantified based on ion transition used for MRM detection as well as their retention times. Representative MRM chromatograms of xanthochymol and isoxanthochymol obtained from standards, *Garcinia* extracts, *Garcinia* extracts spiked with xanthochymol and isoxanthochymol, standard extracts (i.e., extracts in which xanthochymol and isoxanthochymol were absent) and standard extracts spiked with xanthochymol and isoxanthochymol are shown in Figs. 7 and 8.

The validated method was employed for analysis of xanthochymol and isoxanthochymol in the extracts of fruit rinds,

Table 3
A summary of the concentrations (mean ± S.D.) of xanthochymol and isoxanthochymol in seven samples of *Garcinia indica* (GI) and two samples of *Garcinia cambogia* (GC)

Number	Sample	Source	Xanthochymol (ng/ml) Mean ± S.D.*	Isoxanthochymol (ng/ml) Mean ± S.D.*
1	GI fruit rinds[a]	B	113.66 ± 0.75	23.28 ± 0.68
2	GI fruit rinds[a]	D	23.07 ± 0.68	6.98 ± 0.59
3	GI stem bark[a]	D	7.89 ± 0.63	3.31 ± 0.29
4	GI seed pericarp[a]	D	7.73 ± 0.41	3.42 ± 0.31
5	GI leaves[a]	D	2.52 ± 0.28	2.56 ± 0.16
6	GI fruit rinds[b]	B	nd	6.71 ± 0.30
7	GI fruit rinds[c]	B	nd	nd
8	GC fruit rinds[a]	K	4.93 ± 0.21	nd
9	GC fruit rinds[b]	K	nd	nd

B: sample collected from Bangalore, India; D: sample collected from Dapoli (Maharashtra), India; K: sample collected from Kerala, India.
[a] MeOH extract.
[b] Aqueous extract.
[c] *n*-BuOH extract.
* $n = 6$.

stem bark, seed pericarp, and leaves of *G. indica* and in the fruit rinds of *G. cambogia*. The results are summarized in Table 3.

4. Conclusions

A validated, sensitive and selective LC-MS/MS method for identification and quantification of two biologically active molecules xanthochymol and isoxanthochymol from *Garcinia* species has been developed. The following advantages such as shorter analysis time for LC and low LOQ of 1.0 ng/ml for xanthochymol and 0.5 ng/ml for isoxanthochymol make the method attractive for the analysis of the above two compounds in different plant parts of *Garcinia* species. This is the first report of LC-MS/MS analysis of xanthochymol and isoxanthochymol in the Garcinia species. The results of validation indicate that the method can be considered for the screening of other extracts for the analysis of the above two molecules.

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