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EVALUATION OF ANTIOXIDANT ACTIVITY AND VALIDATED METHOD FOR ANALYSIS OF POLYPHENOLS FROM NON-EDIBLE PARTS OF INDIAN TROPICAL FRUITS BY USING MICROWAVE ASSISTED EXTRACTION AND LC-MS/MS

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ABSTRACT

Antioxidant activity and polyphenols were determined by Microwave assisted extraction technique (MAE) from non- edible parts of Indian tropical fruits. Antioxidant activity was performed using FRAP, ABTS and DPPH assays. Nine polyphenols were quantified by LC-MS/MS. Results showed that skin extract of *A. comosus* contained highest value for all antioxidant assays. Polyphenolic compounds were found highest in *Carissa carandas* pomace. In all the obtained extracts rutin was present as major flavonoid. Method was optimized and validated for the analysis of polyphenols as per the ICH requirement for analytical methods. To validate the matrix effects repeatability, reproducibility, recovery and overall uncertainty were calculated for the six matrices at 0.025, 0.050 and 0.100 μg gm^{-1} concentration. Recovery was ranged between 80.2 to 107% with RSD less than 15.0 %. Results suggest that MAE is a good alternative for extraction of polyphenols and provide significant advantages in terms of extraction efficiency and timesavings.

KEYWORDS: **LC-MS/MS, MAE, Polyphenols, Food industrial wastes and Validation.**

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INTRODUCTION

In recent decades, rapidly growing body of literature covers role of plant phytochemicals and their potential effect on human health. Phytochemicals are naturally occurring bioactive substances, with various pharmacological action and therapeutic application. Literature serves a huge study that phenolic substances are the main phytochemicals with antioxidant properties found in higher plants. Intake of these substances daily in adequate amount can play an important role in preventing cancer, cardiovascular and various chronic diseases [1-3]. Huge amount of by- product or wastes materials of fruits & vegetables are generated by food processing industries, which represents a major disposal problem. Research shows that this disposed material has been evaluated with a promising source of medicinal properties and becoming subject of recent review [4]. Sometimes it has been found that by-products could be more valuable than main products because important phytochemical and other bioactive compounds are present in these residues. These by-products or wastes material are generally being underutilized in developing countries. For proper utilization of this wastes it makes more necessarily to develop improve methods for extraction of these important phytochemicals rapidly, efficiently and inexpensively [5]. Many conventional extraction systems could diminish the activity of the phenolic antioxidants because they are very sensitive to specific solvents and temperature. Several extraction techniques and solvents are used for obtaining these compounds from plant sources. Generally, extraction technique includes; Soxhlet extraction (SE), ultrasound assisted extraction (UAE), microwave assisted extraction (MAE) and supercritical fluid extraction (SCFE). Among all, Microwave assisted extraction has been widely used as a sample preparation technique in different analytical fields including environment and food & agriculture. Many reports have been published on application of microwave heating for extraction of organic compounds and pesticide residues but a few publications do exist for extraction of phytoconstitutents by microwave-assisted extraction. Studies demonstrated that the microwave-assisted extraction (MAE) increased the recovery of trace residues in vegetables, fruits, coffee, tea, and beans [6, 7]. It takes less time, less solvent, enables batch process and can be automated for simultaneous extraction at high pressure and temperature in a secured closed vessel system [8]. The main objective of this work was to demonstrate the utility of microwave - assisted extraction (MAE) technique for estimation of antioxidant activities and polyphenols from non-edible parts of Indian tropical fruits. Method was also optimized validated and calculated uncertainty measurement (UM) for the analysis of polyphenols as per the International conference on Harmonization (ICH) requirement for analytical methods.

MATERIALS AND METHODS

Plant material & Chemicals

Samples *Carissa carandas* (pomace), *Ananas comosus (*Skin), *Artocarpus lachoocha* (pomace), *Litchi sinensis* (seeds), *Grewia asiatica* (pomace) and *Beta vulgaris* (pomace) were obtained from local market Delhi, India in 2011. Skin & seeds were collected manually by cutting with a stainless steel knife in small pieces and pomace was collected after extracting the juices. Samples were freezed and vacuum dried. A powder was obtained by crushing & grinding, then powdered in cyclotech mill using 500-mesh size to get a fine powder (DM), and stored at 4 ^{0}C for further analyses. HPLC and AR grade analytical solvents were used in the analysis and purchased from RFCL, Delhi, India). The reference standards were obtained from Sigma- Aldrich (Sigma- Aldrich, St. Louis, MO, USA**)**. Apparatus; Blender (Inter science, Japan), Vortex mixture (Jain Sci. India), Centrifuge, Sigma 2-16 K (SV Instrument, Delhi, India) and rotary evaporator (caterpillar, Prama Inst, India) was used in the evaporation.

Sample preparation

Microwave assisted extraction (MAE)

Microwave assisted extraction was carried out using Microwave reaction system (Multiwave 3000Solv, Anton Paar, Europe). 1 gm of powdered sample was accurately weighed and added 20 ml of solvent aqueous methanol (methanol: water, 80:20 v/v; AME), aqueous acetone (acetone: water, 80:20 v/v; AAE) and mix solvent (Ethanol: Hexane: water 80:10:10 v/v; MSE). Samples were extracted using the following microwave program: 2 min ramp from 100 to 300W, 3 min hold at 300W, 2 min ramp from 300W to 100W, 2 min hold 100 W and after cooling the vessels contents. Total nine compounds were used for quantitative analysis of polyphenols in all samples. Extracted samples were centrifuged at 4,000 rpm & 4 ^{0}C, filtered through a 0.45μm nylon syringe filter (Millipore) and evaporated with a rotary evaporator (Nutronix, Jain Brothers India) below 50 ^{0}C under nitrogen before colorimetric and chromatographic analysis.

Antioxidant activities

Ferric reducing activity power (FRAP) assay

The FRAP assay was carried out according to the method of Stratil using freshly prepared FRAP reagent [9]. Extracts were mixed with FRAP reagent and measured at 593 nm after 40 min. The results were calculated as trolox equivalent (TE μM g^{-1} DM).

ABTS radical scavenging assay

The ability of the test samples to scavenge $ABTS^{\cdot +}$ radical cation was compared to trolox standard [10]. The $ABTS^{\cdot +}$ radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and incubating for 12–16 h. The absorbance of the $ABTS^{\cdot +}$ solution was equilibrated to 0.70 ± 0.02, then extracts were mixed with 10 μl of the test sample (0.05–10 mg ml^{-1}) and the absorbance was measured at 734 nm after 6 min. The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the trolox equivalent antioxidant concentration (TEAC). To calculate the TEAC, the gradient of the plot for the sample was divided by the gradient of the plot for trolox [11].

DPPH radical scavenging assay

The stable DPPH was used for determination of free radical scavenging of extracts according to the method Chang [12]. Briefly, 10 μl of sample (0.05-10 mg ml^{-1}) were mixed with 90 μl of 50 mM Tris–HCl buffer (pH 7.4) and 200 μl of 0.1 mM DPPH ethanol solution. After 30 min of incubation at ambient temperature, the absorbance was taken at 517 nm. Catechin was used as a positive control. The inhibition ratio (%) was calculated according to the equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] X100.

Polyphenols analysis

Instrumentation

Chromatographic system was consisted of an Agilent 1100 series HPLC instrument and 6460 triple quad MS detector. Separation was carried out on a C18 column (4.6 mm × 100 mm × 5 μm, Agilent Technology) at a flow rate of 0.8 ml min $^{-1}$, with a two solvent mobile phase (eluent A = 10mM ammonium acetate and 1% Acetic acid in water; eluent B = 1% Acetic acid in methanol). The eluent gradient used for all extracts was as follows: 0–3 min, 15–40% A; 3-5.5 min, 40-90% A; 5.5–9 min, 90% A; 9–9.5 min, 90-15% A; 9.5-10 min, 15% A. Sample injection volume was 20 μl.

Mass spectrometry

For ESI-MS/MS (Agilent Jet stream) fragmentation the following instrumental parameters were used: Gas Temp, 350 ^{0}C; Gas flow, 10 l min^{-1}; Nebulizer, 50 psi; Sheath gas temp, 400 ^{0}C; Sheath gas flow, 10 l min^{-1}; Capillary voltage, 4000 V in negative ionization mode [13]. Data was acquired by Agilent triple quad LC-MS MassHunter Workstation. Quantitation was based on external standardization by employing calibration curves in the range of 1–50 ng ml^{-1} based on the peak area calculated from selected ion chromatograms of the corresponding $[M-H]^{-}$ ion and results were expressed as μg per gm DM. The calibration graph of each polyphenol was constructed using seven different concentrations of standards mix solution. Compounds extracted by MAE were used to determine the concentration of polyphenols.

Validation & Estimation of Uncertainty Measurement

Extraction method was also studied for sensitivity, linearity, precision, recovery, accuracy and selectivity as per the ICH requirements for analytical methods. The sensitivity of method was evaluated by determining the limit of detection (LOD) and limit of quantitation (LOQ) by measuring the magnitude of analytical background by injecting the blank. In this study, LOD was determined by injecting a series of solutions until the height of the peak signal to baseline noise ratio (S/N) was 3:1, while limit of quantitation (LOQ) values were taken at S/N 10:1. The linearity of the method was investigated by spiking blank samples with known concentrations of standard at five-concentration level. In order to check the accuracy of extraction method was evaluated through recovery study by spiking the samples with all reference standard at three different concentrations (0.1, 0.05 and 0.025 μg gm^{-1}) and three replicates for each concentration were performed together with a calibration curve and established the repeatability (intra-day precision) and intermediate precision (inter-day precision). The precision was represented by the intra- and inter-day relative standard deviation (%RSD). The within-day accuracy and precision were determined with three replicate on a single day, while the between-day accuracy and precision was carried out over three consecutive days. Results with less than 20% relative standard deviation (RSD) and 70-120% of recovery were accepted as satisfactory. The different spiking levels were carried out to reflect the sensitivity of the detector towards the different concentration. The accuracy/recovery was determined as the mean of the measured value relative to the theoretical spiked values and was reported in percentage (%). The measurement of uncertainty is a parameter associated with the result of measurement that characterizes the dispersion of the values that could reasonably be attributed to the Measurand. In this experiment it was performed as per the ISO guide to the expression of uncertainty in measurement (GUM, 1993), under the repeatable and reproducible conditions for 9 polyphenols in 6 commodities.

Statistical analysis

All the data was reported as mean ± SD. Analysis of variance was performed using the ANOVA procedure. Statistical analysis performed according to SAS software. Differences at $P < 0.05$ were considered statistically significant.

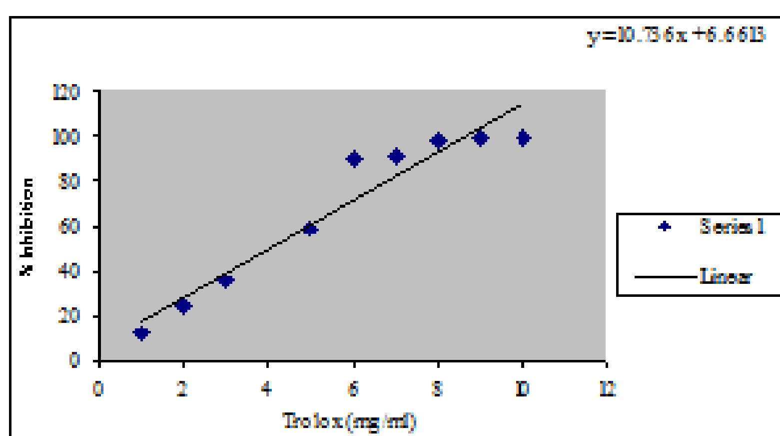
RESULTS AND DISCUSSION

Antioxidant activities

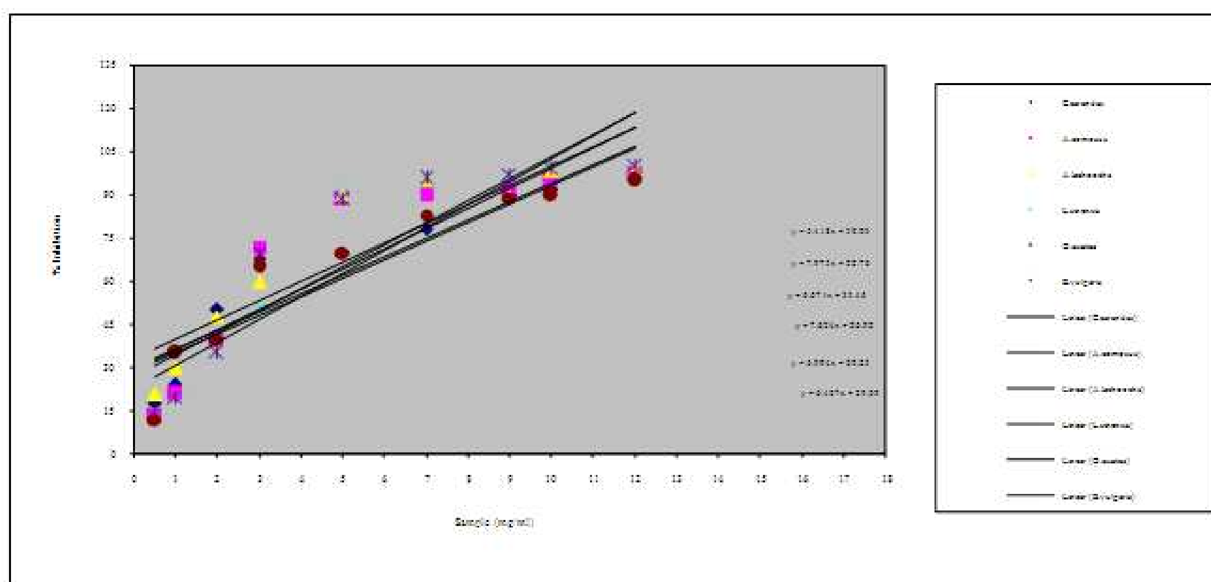
Antioxidant compounds like phenolic acid, polyphenols and flavonoids scavenge free radicals and thus inhibit the oxidative mechanisms that lead to degenerative disease. Different antioxidant compounds may act through different mechanisms and one method alone cannot be utilized to fully evaluate the antioxidant capacity. Therefore, different antioxidant assays with different approach was carried out. The analysis of different antioxidant assays observed that results were high to low in respect of their polarity. Among all tested extracts, methanol extract of *A. comosus* contained the highest level for FRAP assay (32.00 ± 0.31, TE μM g^{-1}), TEAC value (1.50 ± 0.25 DM) & DPPH radical scavenging activity as IC_{50} (0.010 ± 0.12 mg ml^{-1}. Pomace extract of *A. lachoocha* and seeds of *L. sinensis* were found the weakest residues for the FRAP assay. However, all the extracts were found good for DPPH radical scavenging activity besides, *Grewia asiatica* (0.78 ± 0.09mg ml^{-1}). In previous study, DPPH radical scavenging activity for *Litchi sinensis* seeds was recorded as 48.9 ± 0.25 in 50% ethanol extract in 100 μg ml^{-1} [14]. In another study of *Litchi sinensis* seeds, Dhan (2011) reported DPPH IC_{50} value as 0.068 mg ml^{-1} in 50% methanol extract [15]. This value was significantly high in this experiment when extraction was made by microwave reaction system. Rakholiya et al., (2011) carried out the study on *A. comosus* skin extract and found DPPH IC_{50} value less than 1000 μg ml^{-1} in methanol extract by conventional method [16]. DPPH IC_{50} value for *A. comosus* methanol extract was to be 0.010

± 0.12 mg ml^{-1} by microwave extraction in this experiment. Our study shows that the results obtained by MAE in methanol extract were comparatively higher than reported earlier by conventional extraction. In conventional extraction heating depends on conduction- convection phenomenon and much of the heat energy being lost in the environment, whereas in MAE heating occurs in the targeting and selective manner with particularly no heat being lost to the environment as the heating occurs in a closed system. The antioxidant activities for all samples measured in different solvents are listed in Table-1. TEAC value of all extracts was calculated from the decolorization of $ABTS^{\cdot+}$. Interaction with the standard trolox or extracts suppressed the absorbance of the $ABTS^{\cdot+}$ radical cation and the results, expressed as percentage inhibition of the absorbance (Figure-1).

Figure 1
Total antioxidant activity of trolox and extracts.



Trolox mg ml^{-1} (a)



Sample mg ml-1 (b)

Effect of (a) Reference compound trolox (b) Extracts (AAE) by Microwave digestion and on decolorization of ABTS radical cation. The percentage inhibition was plotted against the concentration of sample. All data are expressed as mean ± S.D. (n = 3)

Table-1
Antioxidant activities

Commodity	Antioxidant activities								
	FRAP as TE μM g^{-1} (DM)			Total antioxidant activity (TEAC) value (DM)			IC_{50} of DPPH radical (mg ml^{-1} extract)		
	MSE	AME	AAE	MSE	AME	AAE	MSE	AME	AAE
C. carandas	20.20 ± 0.28	22.50 ± 0.10	12.60 ± 0.25	1.10 ± 0.12	1.20 ± 0.18	0.45 ± 0.25	0.063 ± 0.11	0.058 ± 0.21	0.070 ± 0.30
A. comosus	27.75 ± 0.22	32.00 ± 0.31	25.00 ± 0.58	1.30 ± 0.11	1.50 ± 0.25	1.25 ± 0.14	0.015 ± 0.14	0.010 ± 0.12	0.024 ± 0.10
A.lachoocha	8.50 ± 0.25	10.75 ± 0.40	7.50 ± 0.60	1.05 ± 0.18	1.24 ± 0.20	1.02 ± 0.69	0.065 ± 0.14	0.060 ± 0.26	0.075 ± 0.15
L. sinensis	8.74 ± 0.10	9.20 ± 0.56	8.00 ± 0.27	1.22 ± 0.10	1.42 ± 0.04	1.20 ± 0.50	0.053 ± 0.17	0.049 ± 0.25	0.060 ± 0.10
G. asiatica	15.30 ± 0.40	16.87 ± 0.12	10.50 ± 0.36	1.23 ± 0.25	1.30 ± 0.21	1.14 ± 0.41	0.70 ± 0.07	0.67 ± 0.05	0.78 ± 0.09
B. vulgaris	8.20 ± 0.40	12.80 ± 0.29	6.50 ± 0.54	1.04 ± 0.13	1.19 ± 0.20	1.00 ± 0.31	0.018 ± 0.36	0.010 ± 0.11	0.030 ± 0.13

A All data are expressed as mean ± SD (n = 3)

Polyphenols

The concentrations of six major polyphenols; flavanols, hydroxybenzoic acids, ellagitannins, hydroxycinnamic acids, and flavonols were quantified by LC-MS/MS. These polyphenols have been reported for their broad spectrum of biological and antioxidant activities. The influence of the extraction solvents is assessed in Table-4. For all compounds, [M-H] - (Frag. MS_2 *m/z*), Fragmentor (V), collision energy, retention time (min) and LOD are evaluated in Table - 2. Each analyte was scanned at both polarity (positive and negative) and ion mode was chosen on the basis of best response. Calibration curves for all compounds were linear and were ranged between R^2 = 0.9929 – 0.9982. The highest amount of all polyphenols was found highest in *Carissa carandas* pomace. Chlorgenic acid, Rutin and Quarcetine were not detected in *L. sinensis* seeds, while myrecetin was present in lower amount. Ascorbic acid was in the highest amount in *Ananas comosus* skin extract (95.5 ± 0.25 µg gm^{-1}). In all the extracts rutin was present as major flavonoid followed by quercetin, catachein and myrecetin. Some polyphenolic compounds could not be detected in acetone extract. *Grewia asiatica* had relatively low level of all polyphenols. Amount of total polyphenols by MAE in methanol extract for all residues was ranked in the following descending order: *C. carandas*> *A. comosus*> *A. lachoocha*> *L. sinensis*> *B. vulgaris*> *G. asiatica*. No specific literature was found for quantification of polyphenols in selected matrixes; only a few reports are existed. Gallic acid, procyanidin B2 (-), gallocatechin (-), epicatechin (-) and epicatechin-3- gallate were identified in 50% ethanol extract in *Litchi sinensis* seeds [14]. Furthermore, in another study of *Litchi* seeds ellagic acid and p coumaric acid were quantified as 46.4 µg gm^{-1}, 70.1 µg gm^{-1} respectively [15]. In our study results for ellagic acid was slightly higher with previous reported result. Figure 2(a) and 2(b) show the TIC (Total ion chromatogram) & MRM (Multiple reaction monitoring) of all extracted compounds in *A. comosus* skin in AME.

In our study polyphenols extracted by methanol was slightly higher than mix solvent but was ≈ 2× higher than that extracted by acetone. Phenolic compounds are commonly more soluble in aqueous polar organic solvents. Thus, the preferable solvents those were used in our study were aqueous mixtures of methanol, ethanol, and acetone. Due to differences in geographical, extraction methods, solvents, unit of measurements applied in the previous literature, fair comparison cannot be made with this study. Polyphenols are not only good antioxidant but also, been shown to be a potent as anticarcinogen and antimutagen. The correlation between polyphenols and antioxidant activity has been widely studied by different researchers[17, 18]. The results obtained in our study shows good correlation between polyphenols (measured by LC-MS/MS) and FRAP & ABTS assays as 0.96 to 0.65, $P < 0.05$ indicating that these polyphenols are important contributors of antioxidant potential of extracts while, DPPH radical scavenging activity was negatively correlated. Recent research on antioxidant properties of plant has been accepted that higher intake of natural antioxidants containing polyphenols is associated with long term health benefits. The results presented in this study for selected matrices offer possible avenues towards health promotion and might be useful in the development of raw materials of medicine and functional food formulations.

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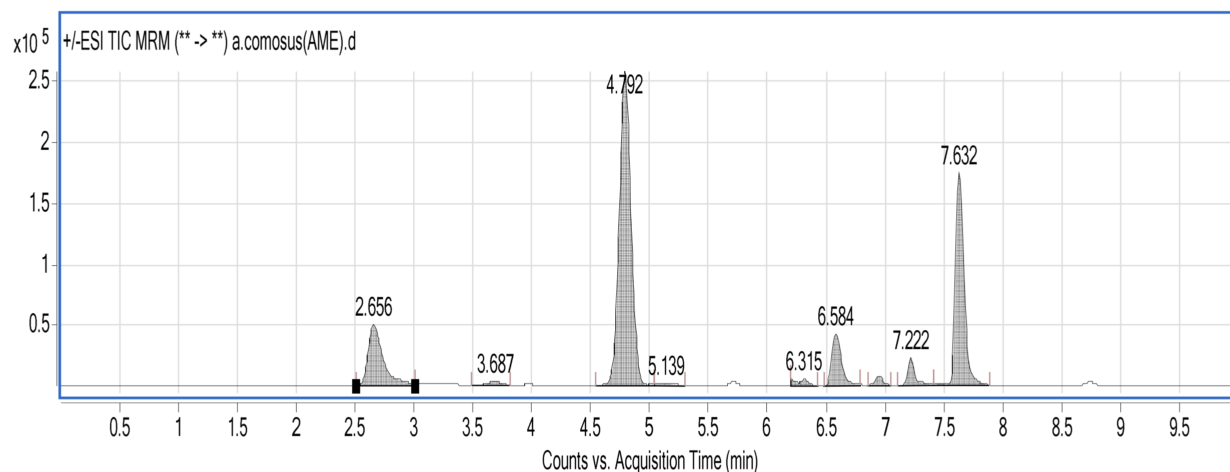
Table 2- Chromatographic condition

Compounds	Pre Ion	Pro Ion	Frag (V)	CE (V)	RT (min)
Ascorbic acid	175	115	75	4	4.755
Gallic acid	169	125	65	12	2.664
Ellagic acid	301	151	120	20	7.635
Chlorogenic acid	353.3	191.3	90	12	4.793
P-Caumaric acid	163	119	90	10	6.592
Catechin	289	245	125	10	4.636
Rutin	609	300	145	35	6.946
Myrecetin	317	151	120	20	7.227
Quarcetine	301	179	125	12	7.633

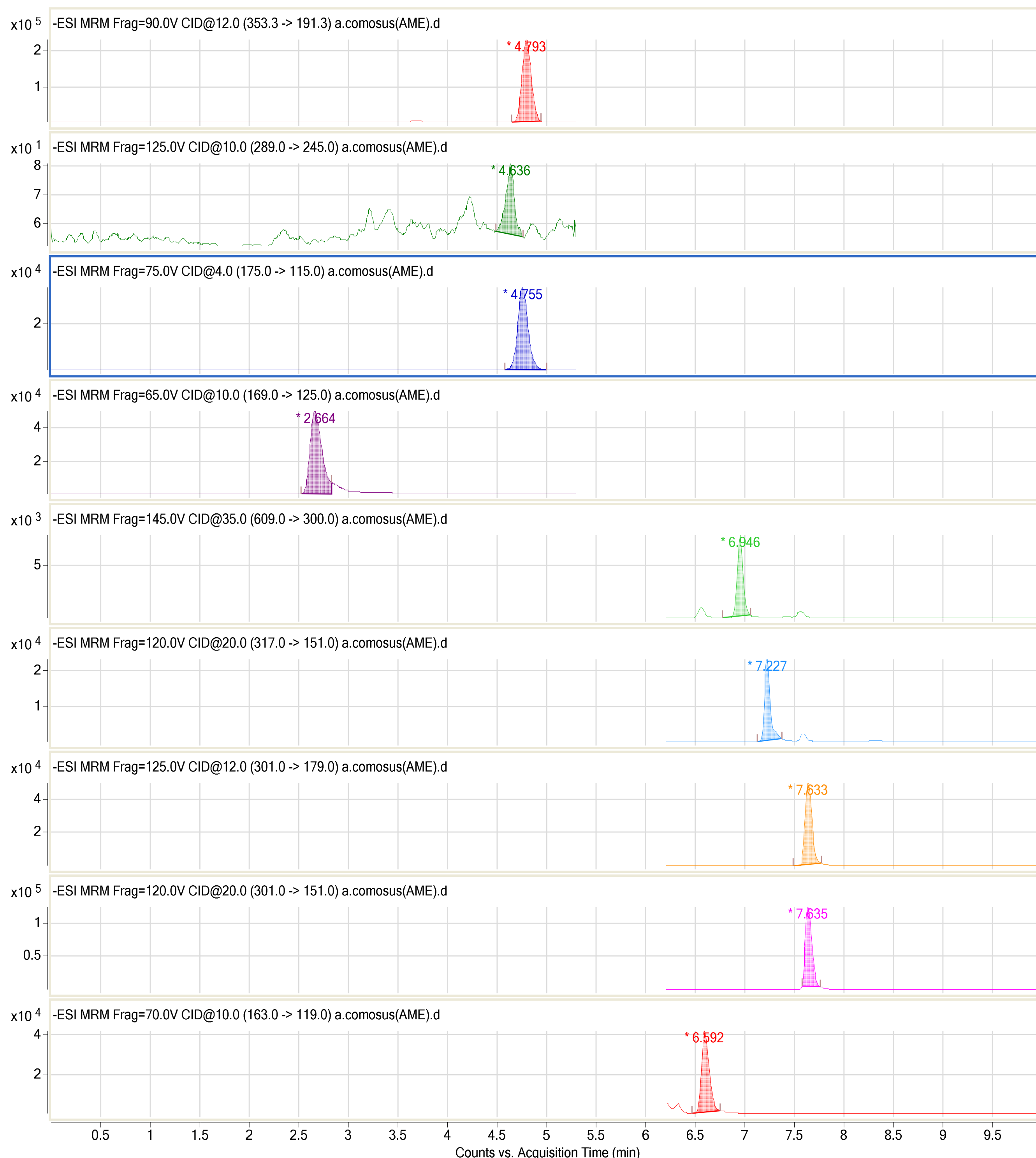
Pro ion-product ion, Frag- Fragmentor

Figure 2

(a) TIC (Total ion chromatogram) of A.comosus in AME by MAE



(b) *MRM (Multiple reaction monitoring system) by LC-MS/MS*



Method optimization

Optimization of Microwave Extraction

In Microwave assisted extraction, factor that contributes to the efficiency of solvent extraction is type of solvent, temperature, volume of solvent, number of extraction step, particle size and time employed. A correct choice of solvent is the fundamental for obtaining an optimal extraction process. It has been established that when solvent

polarity is modified by the addition of water, increased yields are obtained. Small amount of water in the extracting solvent can penetrate easily into the cells of plant matrix and thus increases the mass transfer of the active constituents into the extracting solvent [19,20 &21]. Polarity of solvent is also very much important in microwave extraction because solvent with high dielectric constant can absorb more microwave energy. Most of the time, the sample is immersed in a single solvent or mixture of solvents that absorb microwave energy strongly [22]. Some solvents are known good extraction solvent but not a good absorber of microwave energy. However, some solvents are known for good absorber but not a good extraction solvent. Therefore mixture of solvent in different ratio is used for MAE process to produce optimum results. Another important aspect is the compatibility of the extracting solvent with further chromatographic analytical steps. The solvent selected in our study for the extraction were due to their good microwave absorbing property and high selectivity towards the analytes of interest. Extraction was carried out as above stated power programme to avoid over- heating and get maximum recovery. Temperature obtained during the extraction was measured 75-80 ^{0}C into the vessels and this temperature has been reported adequate for the determination of polyphenols [23]. High power may be involved the risk of thermal degradation. Low power with longer exposure could be wise approach and provides more yields. At higher temperature rapid rupture of cell may also take place, as a result together with the desired analyzes impurities are also leached out into the solvent that affects the purity[24]. The principle of heating using microwave energy is based on the direct effect of microwave on molecules by ionic conduction and dipole rotation. Extraction rate improves proportionally with the degree of grinding. Fine powders can enhance the extraction by providing large surface area, which gives better contact between the sample matrix and the solvent. Usually extracted materials are become in a range of 100 µm-2 mm. For this purpose grinded sample were powdered in cyclotech mill using 500-mesh size to get a fine powder. Volume of extracting solvent is also a critical factor. A higher ratio of solvent volume may yield lower recovery in MAE. In many applications a ratio 10:1 to 20:1(ml/mg) was found to be optimum [25]. Parameter for Microwave extraction was designed to consider of all said points to obtain optimum results.

Optimization of Chromatographic condition

In order to check the reproducibility of retention time and peak area a standard mixture solution was also analyzed at three different concentrations under the optimum conditions in the experiment. The within-day accuracy was determined with three replicate on a single day, while the between-day accuracy was carried out over three consecutive days. The relative standard deviation (RSD) of the peak area was ranged 0.5-2.5 for intra-day and 2.1 - 3.6 for inter-day analysis, while and relative standard deviation (RSD) of retention time was 0.05-0.4 for intra -day and 0.25-1.16 for inter-day analysis (RSD < 2.0%).

Validation & Estimation of Uncertainty Measurement

Extraction method was also studied for sensitivity, linearity, precision, recovery, accuracy and selectivity as per the ICH requirements for analytical methods to check the method efficiency [26]. In this study, the solvent with the highest extraction efficiency in the microwave solvent extraction was used for validation study.

Sensitivity & Linearity

Optimized LC-MS/MS analytical conditions were used for the determination of polyphenols as per the described method [13]. The LOD is the lowest concentration of the analytes in a sample, which can be detected but not necessarily quantified. The LOQ is the lowest concentration of the analytes in a sample, which can be quantified with an acceptable degree of accuracy and precision. The LOD was established for a signal to noise ratio of 3 and verified (analyzing

replicates of different samples) as three times the standard deviation (SD) of the obtained noise (LOD=3×SD). The LOQ was defined as the analytes concentration resulting in S/N of 10 and verified by afore-mentioned procedure applied for LOD. Finally LOQ was taken as the concentration with the acceptable precision & accuracy of the measurement and were analyzed for repeatability and recovery study. LOD & LOQ for all compounds are in table-3 and were in the range of 0.001- 0.0025 µg gm^{-1} & 0.011 - 0.030 µg gm^{-1} respectively. The linearity of the method was obtained by least-squares linear regerration analysis of the peak area versus analytes concentration, using seven concentration levels in triplicates. The correlation coefficient (r^2 >0.99) is shown in Table-3.

Table 3
Validation parameter of method

Compounds	r^2	LOD (µg gm^{-1})	LOQ (µg gm^{-1})	% Recovery ±SD	Intra-day Precision(%RSD)	Inter-day Precision(%RSD)	RSD (%) for retention time	RSD (%) for retention time	RSD (%) for Peak Area	RSD (%) for Peak area	± UM
							Intra-day	Inter-day	Intra-day	Inter-day	
Ascorbic acid	0.9957	0.0025	0.030	82.0 ± 3.5	11.9	12.7	0.36	1.12	2.3	2.7	22.3
Gallic acid	0.9929	0.0020	0.016	95.2 ± 2.7	10.0	11.1	0.40	1.04	0.6	2.1	20.5
Ellagic acid	0.9946	0.0018	0.012	92.0 ± 4.1	7.2	9.5	0.20	1.02	1.0	2.5	21.6
Chlorogenic acid	0.9962	0.0019	0.016	80.2 ± 2.6	8.5	10.5	0.15	0.96	2.8	3.5	22.0
P-Caumarric acid	0.9982	0.0011	0.016	103 ± 7.7	5.3	9.9	0.09	1.16	1.1	2.9	24.6
Catechin	0.9963	0.0010	0.020	97.0 ± 6.4	11.2	12.0	0.05	1.16	2.5	2.9	23.4
Rutin	0.9937	0.0010	0.015	80.4 ± 3.9	8.6	10.1	0.10	0.25	1.0	2.1	22.9
Myrecetin	0.9945	0.0015	0.029	105 ± 3.1	6.9	7.2	0.15	0.45	0.5	2.2	24.1
Quarcetine	0.9933	0.0011	0.011	107 ± 4.9	8.1	9.5	0.12	0.80	1.4	2.5	22.6

r^2 (coefficient of regression) ,LOD (µg gm^{-1}) (Limit of detection), LOQ (µg gm^{-1}) (Limit of quantification), data are expressed as mean (Recoveries (%) RSD %, n=3 for intra day and n=3 for inter day analysis). Intra-day precision (Repeatability)& Inter-day precision expressed as pooled RSD and overall uncertainties expressed as percent (k=2) calculated at LOQ level of the investigated pesticide using the optimised MAE-LC-MS/MS method.

Precision, Recovery & Accuracy

The repeatability of an analytical method refers to the use of procedure within a laboratory over a short period of time, carried out by the same analyst with the same equipment. The inter-day accuracy and repeatability were assessed, at three concentration levels with three replicates for each concentration on the same day. While the intermediate precision was based on the mean repeatability values of a set of spiked samples at three concentration levels and analysed daily for a period of 3 days. The recovery and RSD of the method were assessed by analyzing different samples (n=6) for inter day (n=3) and intra day (n=3) analysis. The recovery was in the range of 80.2-107 % with R.S.D <15%. The values show good precision (RSD <20%, recovery > 80%) in all tested matrices (in agreement with the ICH requirements for analytical methods).

Selectivity

The selectivity of method was assessed by comparing the chromatograms obtained with or without the analytes in the blank samples. Matrices with each analyte were injected separately to ensure that no interfering peaks with the same retention time were present.

Overall uncertainties

Due to difficulty in calculating the individual uncertainty contributions a “bottom up” procedure was followed as proposed by the ISO guide (GUM, 1993) and the different contributions were grouped as recommended by the EURACHEM/CITAC guide (EURACHEM, 1995) [27, 28]. The contributions in the MAE–LC-MS/MS method can be grouped in three terms, permitting the calculation of the overall uncertainty according to the following equation:

$$Ur = r \times k \sqrt{(u\,(CRM))^2 + (u\,(Rep))^2 + (u\,(Bias))^2}$$

The first term (*u*CRM) corresponds to the relative uncertainty from the certified reference material used for calibration and the subsequent uncertainties introduced by the balance, volumetric material, etc. during weighing and diluting to the final concentration. The second term (*u* (Rep)) corresponds to the relative uncertainty of contribution due to the precision of the method, also called repeatability uncertainty, which gives a value for the standard uncertainty due to run-to-run variation, day-to-day variation, analyst-to analyst variation and commodity-to-commodity variation of the overall analytical process. (*u* (Bias)) is the relative uncertainty due to *bias* i.e. corresponds to the tolerance that each laboratory establishes for their internal quality controls of the analytical procedure, investigated during the in house validation study using spiked samples (homogenized sample were split and spiked). Finally, *k* and r are the coverage factor and reported result respectively to expand the uncertainty to the desirable level of confidence with desirable units of the measurement. The second and third terms are generally the most important contributions to the overall uncertainties. In the present work, the overall uncertainties were calculated at 0.025, 0.05 and 0.100 µg gm^{-1} level. The *u*CRM was calculated by taking into account of all the dilution steps and the uncertainties from the CRM and all the volumetric material and balances used to prepare the calibration standards. The second term *u*Rep was calculated from the n=6 results (each sample) from the experiment performed under repeatable and reproducible conditions. The third term was calculated considering mean recovery of samples with recovery 80.2% to 107% with RSD less than 20.0 %, tolerance that the laboratory accepts as a maximum for the verification of the daily analysis. Finally, a coverage factor k = 2 was used for a confidence interval of 95% as (n =6). As shown in Table 2, the uncertainties calculated are with in 25%, which is a typical value for a chromatographic method in a routine quality control laboratory. In our case, the tolerance was stated as 25% for practical purposes; nevertheless, the uncertainties of the method can be reduced with a more exigent tolerance in the daily verification by doing the study at higher concentration.

Table 4
Quantification of polyphenols by LC-MS/MS

Commodity	Extraction Solvent	AA	GA	EA	CLA	PCA	CT	RU	MY	Q
C. carandas	MSE	75.8±0.05	53.5±0.04	40.9±0.04	41.6±0.04	39.9±0.01	0.52±0.01	30.5±0.02	ND	23.4±0.02
	AME	82.5±0.17	59.0±0.09	43.1±0.01	52.6±0.03	42.1±0.07	1.50±0.04	34.7±0.09	0.35±0.17	26.7±0.01
	AAE	65.6±0.04	49.5±0.15	39.1±0.01	40.4±0.07	30.0±0.06	ND	28.4±0.12	ND	20.9±0.18
A. comosus	MSE	95.5±0.02	18.0±0.08	20.6±0.16	50.5±0.01	42.2±0.07	1.25±0.04	22.2±0.20	0.56±0.08	10.8±0.02
	AME	96.2±0.01	20.6±0.04	22.3±0.08	55.9±0.04	45.5±0.01	2.59±0.07	28.8±0.16	1.25±0.04	12.2±0.07
	AAE	83.9±0.07	16.5±0.00	19.0±0.04	40.2±0.02	39.9±0.11	ND	20.0±0.13	0.50±0.17	06.2±0.17
A. lachoocha	MSE	15.5±0.02	55.5±0.05	14.9±0.11	21.5±0.04	22.3±0.18	3.12±0.14	22.9±0.10	0.35±0.08	15.5±0.02
	AME	16.2±0.01	56.2±0.07	15.5±0.08	30.8±0.07	25.0±0.07	5.02±0.08	22.8±0.06	0.90±0.14	16.0±0.02
	AAE	13.9±0.07	51.5±0.02	09.0±0.05	20.9±0.07	19.8±0.06	ND	20.0±0.03	0.05±0.27	10.9±0.11
L. sinensis	MSE	6.05±0.02	13.6±0.02	60.6±0.07	ND	51.5±0.09	21.5 ±0.01	ND	UDL	ND
	AME	9.75±0.04	15.9±0.09	66.0±0.01	ND	55.63±0.11	23.6±0.05	ND	0.55±0.08	ND
	AAE	5.00±0.07	11.9±0.07	57.1±0.01	ND	50.5±0.28	19.0±0.04	ND	ND	ND
G. asiatica	MSE	33.0±0.01	42.2±0.09	33.6±0.17	30.2±0.07	43.5±0.06	1.27±0.14	09.9±0.01	0.29±0.01	12.9±0.03
	AME	34.7±0.05	49.9±0.02	35.0±0.51	32.5±0.01	45.0±0.03	2.78±0.05	11.9±0.01	1.47±0.10	15.4±0.04
	AAE	21.5±0.20	41.9±0.01	27.1±0.11	22.4±0.04	40.5±0.13	UDL	08.5±0.09	ND	09.9±0.13
B. vulgaris	MSE	21.0±0.06	23.0±0.06	20.0±0.07	20.0±0.07	15.2±0.27	7.55±0.05	10.0±0.07	ND	10.5±0.07
	AME	22.2±0.07	25.9±0.08	24.5±0.11	24.5±0.11	19.3±0.08	8.05±0.05	12.1±0.11	0.05±0.17	12.3±0.02
	AAE	15.6±0.05	18.7±0.01	18.2±0.10	18.2±0.10	12.2±0.01	5.50±0.05	09.0±0.18	ND	07.9±0.13

All data are expressed as mean ± SD (n = 3); ND- Not detected; UDL Under detectable level; Results are reported in (µg gm^{-1})
AA- Ascorbic acid, GA-Gallic acid, EA- Elagic acid, CLA-Chlorgenic acid, PCA-P Coumaric acid, CT- Catachein, RU-Rutin, MY-Myrecetine,Q- Quercetin.

CONCLUSION

The level of extractable polyphenols and antioxidant activity was found highest in AME. The results obtained by all experimental study suggest that MAE has a great potential for the extraction of polyphenolic compounds and antioxidant activities from food industrial wastes/by-product due to less impurity, speediness, simplicity, higher yield and more recovery with less RSD. This technique should be favored for routine analysis of polyphenols considering factor such as less time, less solvent and much safer than solvent used in other conventional techniques. Therefore, it can save a lot of time and solvent and brings a higher yield for routine analysis of polyphenols. The information of this study would also be helpful in the development and utilization of food industrial wastes as a food antioxidant or as an antioxidant nutritional supplement.

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REFERENCES

1. Maffei FR, Carini MM, Aldini GBF, Rossoni G, Bombardelli E and Morazzoni P, Diet enriched with procyanidis enhances antioxidant activity and reduces myocardial post-ischemic damage in rats. Life Sci, 64 : 627-634 (1999).
2. Lavelli V, Peri C and Rizzolo A, Antioxidant activity of tomato products as studied by model reactions using

xanthine oxidase, myeloperoxidase, and copper-induced lipid peroxidation. J Agric Food Chem, 48: 1442–1448 (2000).
3. Belen S, Angel LA, Yolanda DG, Gloria Db, Anna PL and Francisco P, Phenolic profiles, antioxidant activity and in vitro antiviral properties of apple pomace. Food Chem, 120: 339-342 (2010).
4. Alonso AM, Guille´n DA, Barroso CG, Puertas B and Garcı´a A, Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content. J Agric Food Chem, 50: 5832–5836 (2002).
5. Lowe ED and Buckmaster DR, Dewatering makes big difference in compost strategies. Biocycle, 36: 78–82 (1995).
6. Negeri BO, Foster GD and Khan SU. Determination of Chlorothalonil Residues in coffee. Toxicol Environ Chem, 77: 41-46 (2000).
7. Pan X, Niu G and Liu H, Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. Chemical engine process, 42: 129-133 (2003).
8. Herbert H, Morias P, PAI´ga SP, Alves A and Santos L, Analysis of PCBs in soils and sediments by microwave-assisted extraction, headspace-SPME and high resolution gas chromatography with ion-trap tandem mass spectrometry. Int J Environ Anal Chem, 86 : 391-400 (2006).
9. Stratil P, Klejdus B and Kuban V, Determination of total content of Phenolic compounds and their antioxidant activity in vegetables–Evaluation of spectrophotometric methods. J Agric Food Chem, 54: 607–616 (2006).
10. Re R, Pellegrini N, Proteggente A, Pannala A and Yang M, Rice-Evans C: Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med, 26: 1231-1237 (1991).
11. Bibhabasu H, Santanu B and Nripendranath M. Antioxidant and free radical scavenging activity of Spondias pinnata. BMC Compl & Alter Med, 8: 63 (2008).
12. Chang ST, Wu JH, Wang SY, Kang PL, Yang NS and Shyur LF, Antioxidant activity of extracts from Acacia confusa bark and heartwood. J Agric Food Chem 49: 3420–3424 (2001).
13. Demiray S, Pintado ME, and Castro LMP, Evaluation of Phenolic profiles and antioxidant activities of Turkish medicinal plants Tilia argentea, Crataegi folium leaves and Polygonum bistorta roots. World Acad Sci Eng Tech, 54: 312-317(2009).
14. Prasad KN, Yang B, Yang S, Chen Y, Zhao M, Ashraf M and Jiang Y, Identification of phenolic compounds and appraisal of antioxidant and antityrosinase activities from litchi (Litchi sinensis Sonn.) seeds. Food Chem, 116: 1–7 (2009).
15. Dhan P, Garim U and Pushpangadana P, Antioxidant Potential of Some Under-Utilized Fruits. Indo-Global J Pharma Sci, 1: 25-32 (2011).
16. Rakholiya K, Kaneria M and Chanda S, Vegetable and fruit peels as a novel source of antioxidants, J Med Plants Res, 5: 63-71 (2011).
17. Jayaprakasha GK, Girennavar B and Patil BS, Radical scavenging activities of Rio Red grapefruits and Sour orange fruit extracts in different *in vitro* model systems. Bioresour Technol, 99(10): 4484-4494 (2008).
18. Kedage VV, Tilak JC, Dixit GB, Devasagayam TPA and Mhatre MA, Study of antioxidant properties of some varieties of grapes (*Vitis vinifera* L.). Crit Rev Food Sci Nutr, 47: 175-185 (2007).
19. Nagendra K, Prasad BY, Shaoyu Y, Yulong C, Mouming Z, Muhammad A and Yueming J, Identification of phenolic compounds and appraisal of antioxidant and antityrosinase activities from litchi (Litchi sinensis Sonn.) seeds. Food Chem, 116: 1–7 (2009).
20. Hariram V, Bhaskar N and Balakrishnan, In Vitro Antioxidant Property of Laticiferous Plant Species from Western

Ghats Tamilnadu, India, Int J Health Res, 2: 163-170 (2009).
21. Kongsuwan A, Suthiluk P, Theppakorn T, Srilaong V and Setha S, Bioactive compounds and antioxidant capacities of phulae and nanglae pineapple. Asian J Food & Agric Ind, Special Issue, S44-S50 (2009).
22. Dominique SN and Qian H, Radical scavenging capacity of Rwandan CTC tea polyphenols extracted using Microwave Assisted Extraction. Pak J Nut, 9: 589-593 (2010).
23. Yuangang Z, Chunying L, Yujie F, Chunjian Z. and Stimulaneous, Determination of catacheine, rutin, quercetin kempaferol and isorhamnetin in extract of sea buckthorn (Hippophae rhamnoides L.) leaves by RP-HPLC with DAD. J Pharm Biomed Analysis 41: 714-719 (2006).
24. Shu YY, Ko MY and Chang YS, Microwave-assisted extraction of ginseosides from ginseng root. Microchemical J, 74: 131-139 (2003).
25. Talebi M, Ghassempour A, Talebpour Z, Rassouli A and Dolatyar L, Optimization of the extraction of paclitaxel from Taxus baccata L. by the use of microwave energy. J Sepacial Sci, 27: 1130-36 (2004).
26. ICH-Topic Q2b. Guidance for industry – Validation of analytical procedures: Methodology. U.S. Department of Health and Human Services (1996).
27. EURACHEM, Quantifying uncertainty in analytical measurements, EURACHEM Secretariat, P.O. Box 46, Teddington, Middlesex, TW110LY, UK(1995).
28. ISO guide to the expression of uncertainty in measurement BIPM, IEC, IFCC, ISO, IUPAC, IUPAP, OIML, Geneva (GUM: 1993).