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UHPLC-DAD method development and validation for simultaneous quantification of 7 phenolic compounds in dietary supplements of sage capsules

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ABSTRACT: The content of the dietary supplements of capsules generated from extracts of medicinal plants in terms of bioactive compounds may not be known. The aim of this study is to do method development and validation for quantification of the phenolic compounds (chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid luteolin-7-o-glucoside, and rosmarinic acid) in capsules of dietary sage (*Salvia officinalis*) supplements by ultra-high-performance liquid chromatography with diode array detector (UHPLC-DAD) to facilitate the quality control. Mobile phase gradient method is optimized in reverse UHPLC mode using aqueous acetonitrile as the mobile and C18 (50*2.1 mm, 1.8 µm) as the stationary phase. Quantitation was done by signal specific integration using diode array detector (DAD). Total run time is 18 minutes. Separation of the phenolic compounds were at 35 °C by employing gradient elution of water: trifluoroacetic acid (100:0.1, v/v) and acetonitrile: water: trifluoroacetic acid (95:5:0.1, v/v/v) as mobile phase A and B, respectively at a flow rate of 0.5 ml/min. The method showed good linearity with correlation coefficient (r^2) that varied between 0.9993 and 0.9997. Limit of detection (LOD) and limit of quantification (LOQ) values were 1.97-18.77 and 6.58-62.56 mg/ml, respectively. Recovery (%) were between 90% and 114% for capsule matrix for all compounds at all levels except for ferulic acid and luteolin-7-o-glucoside. The method is suitable for application in quality control laboratories because of the short run time and simultaneous analysis of phenolic compounds in dietary supplements of sage capsules.

KEYWORDS: Phenolic compounds; dietary supplements; sage; *salvia officinalis*; UHPLC; method development and validation; medicinal plants

1. INTRODUCTION

Recently, there is an increasing demand for natural products obtained from medicinal plants due to the availability and sustainability in addition to lack of sides effects in contrast to synthetic drugs. Therefore, the types and number of commercial natural products in various pharmaceutical dosage forms such as tablets, capsules, pastils, tinctures, syrups have increased. The safety, efficiency and control of herbal products have been drawn attention by health organizations since these products are licensed and regulated as food supplements in some countries and regions [1–4]. Sage (*Salvia officinalis*) is one of the medicinal plants grows in the tropical regions from Asia to the Mediterranean and used in the treatment of diseases related to memory loss [5–7] with proven anti-bacterial, antioxidant, anti-cancer, antifungal, antiviral and anti-inflammatory properties [6,8,9].

Health effects of sage are attributed to its rich phenolic content. According to the literature, the main phenolic compounds found in sage are ferulic acid, caffeic acid, carnosol, chlorogenic acid, p-coumaric acid, luteolin-7-o-glucoside, luteolin-7-glucoronid, luteolin-7-rutinosite, rosmarinic acid, and syringic acid [5-8, 10-24]. There are many dietary supplements of capsules generated from sage extracts. However, the quality control of these products in terms of their phenolic content, which is responsible for the pharmacological effect has not been found in the literature [25]. In addition, the types and amounts of secondary metabolites

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in natural products varied by both external and internal factors including cultivation, processing, and storage, therefore amounts of secondary metabolites are considered as a quality indicator [1,3,26].

Appropriate and robust analytical methods is required for the quality assessment of herbal products [2,4,27]. Secondary metabolites in medicinal plants have been determined either qualitatively or quantitatively using effective analytical techniques such as HPLC, thin layer chromatography, liquid chromatography-tandem mass spectrometry[28]. Phenolic compounds can be estimated using spectrophotometric methods; however, these methods measure total phenolic content rather than accurately analyzing individual phenolic compounds due to interference from other molecules and target analytes in the sample [29]. Preferably, compounds are separated by chromatographic methods before spectrophotometric detection. Therefore, the most ideal analytical technique for quantifying phenolic compounds is high-performance liquid chromatography[30,31]. Other analytical techniques, such as gas chromatography, are not often considered due to the need for derivatization of non-volatile phenolic compounds [28,32]. Powerful characterization technique NMR is not considered for quantitation due to low sensitivity [33].

Phenolic compounds in sage extracts were analyzed by the HPLC method [5, 7–10,13,15,17,19, 22, 23, 34]. Phenolic compounds analyzed by HPLC exhibit significant variation depending on the herbal matrix, and these methods often involve extended run times. There are only a few studies with relatively shorter run times with respect to number and type of phenolic compounds [16,18]. Research on the application of UHPLC, which provides higher resolution and reduced analysis time compared to conventional HPLC using UV-Vis or DAD detectors, remains limited. Mass spectrometric detectors in single or tandem mass spectrometry mode with higher selectivity, accuracy and sensitivity suitable for complex plant matrix are also reported [35–37]. However, LC-MS/MS is a costly technique for research and quality control laboratories due to the high expense of instrument maintenance. On the other hand, the utilization of signal-specific integration with affordable DAD detectors has not been widely explored. Current literature predominantly employs single or a limited number of wavelengths for quantitation of phenolic compounds [7,38], and maximum absorption wavelengths commonly used for supporting the identification [9,16,39]. Furthermore, the quantitation of phenolic compounds in commercial products is not extensively covered in the literature [40].

In this study, the development, and validation of UHPLC method for the simultaneous quantitative analysis of caffeic acid, chlorogenic acid, ferulic acid, syringic acid, p-coumaric acid and, rosmarinic acid and luteolin-7-o-glucoside in dietary supplements of sage capsules and powder were done. The objective of this study is to develop and validate a UHPLC-DAD method that offers significantly shorter analysis times and improved selectivity, overcoming the extended run time limitations commonly seen in most HPLC methods for analyzing phenolic compounds in dietary sage capsules.

2. RESULTS and DISCUSSION

2.1. UHPLC Method Development

Maximum absorbances of caffeic acid, chlorogenic acid, syringic acid, ferulic acid, p-coumaric acid, rosmarinic acid, luteolin-7-o-glucoside, carnosol and butylhydroxytoluene are determined at wavelengths of 324, 322, 276, 324, 310, 330, 336, 286 and 278 nm, respectively. Two C18 column with dimensions of 250x4.6 mm, 5 µm and 2.1x50 mm, 1.8 µm were tested for achieving the ideal separation of the phenolic compounds. Shorter and narrower column with smaller particles size generated relatively faster elution. Experiments were proceeded with injection volume of 1 µl since larger volumes such as 10 µl resulted in column overload and disturbed peaks. Relative retention times of the compounds were determined by running standard solutions of phenolic compounds using mobile phase composition of acetonitrile: water: trifluoracetic acid (50:50:0.1, v/v/v). The separation of the analytes was evaluated by testing the water: acetonitrile: trifluoroacetic acid (90:10:0.1, v/v/v) and acetonitrile: water: trifluoroacetic acid (90:10:0.1, v/v/v), as mobile phase A and B, respectively. Complete gradient program corresponding to method 1 (please see Table 1). While, p-coumaric acid, ferulic acid, luteolin-7-o-glucoside and rosmarinic acid were separated, chlorogenic acid, caffeic acid and syringic acid eluted between 2-5 minutes and carnosol and internal standard are eluted around 10 - 11 minutes demonstrating insufficient separation (Figure 1A). The peak eluted at 9.2 minutes corresponds to an impurity that does not interfere with rosmarinic acid and luteolin-7-o-glucoside eluted around similar retention times because of respective detection wavelengths of these phenolic compounds are 330 and 336 nm. On the other hand, separation of impurity from carnosol and internal standard detected 286 nm and 278 nm, respectively is considered critical. Therefore, both mobile phase composition and gradient

were improved further to separate all the compounds by applying method 2 (Table 1) with mobile phase A of water: acetonitrile: trifluoroacetic acid (95:5:0.1, v/v/v) and mobile phase B of acetonitrile: water: trifluoroacetic acid (95:5:0.1, v/v/v). While syringic acid is separated, chlorogenic acid and caffeic acid are not resolved (Figure 1B). Next, method 3 (Table 1) by using mobile phase A of water: trifluoroacetic acid (100:0.1, v/v) and B of acetonitrile: water: trifluoroacetic acid (95:5:0.1, v/v/v). As a result, separated peaks of caffeic acid, chlorogenic acid and syringic acid are observed at 5.8 min, 6.7 min and 7.9 min, respectively (Figure 1C). Gradient flow elution between flow rates of 0.5 - 0.7 ml/min corresponding to method 4 has also been examined for earlier retention (Table 1). All compounds are eluted earlier compared to method 3, except rosmarinic acid and internal standard (Figure 1D). The identification of the compounds is confirmed by running pure standard of each compound individually for each method. Generally, it is noted that carnosol was neither resolved nor demonstrated an intense signal.

Table 1. Gradient program and other chromatographic parameters. Time (minutes), flow rate (ml/min) and percent composition of mobile phase B (B%).

Method 1			**Method 2**			**Method 3**			**Method 4**		
Time	B%	Flow	Time	B%	Flow	Time	B%	Flow	Time	B%	Flow
0	0	0.5	0	0	0.5	0	4	0.5	0	4	0.7
4	10	0.5	5	0	0.5	8	4	0.5	5	4	0.7
5	10	0.5	6	10	0.5	8.1	15	0.5	5.1	4	0.6
9	75	0.5	7	10	0.5	12	25	0.5	8	15	0.6
9.1	100	0.5	12	80	0.5	13	25	0.5	8.1	15	0.6
12	100	0.5	12.1	100	0.5	13.1	100	0.5	12	25	0.6
12.1	0	0.5	14	100	0.5	16	100	0.5	13	25	0.5
16	0	0.5	14.1	0	0.5	16.1	4	0.5	13.1	100	0.5
			16	0	0.5	18	4	0.5	16	100	0.5
									16.1	4	0.7
									18	4	0.7
Mobile Phase A (water:acetonitrile: trifluoroacetic acid, 90:10:0.1, v/v/v)			Mobile phase A (water: acetonitrile: trifluoroacetic acid, 95:5:0.1, v/v/v)			Mobile phase A (water: trifluoroacetic acid 100:0.1, v/v)					
Mobile phase B (acetonitrile: water: trifluoroacetic acid, 90:10:0.1, v/v/v)			Mobile phase B acetonitrile: water: (trifluoroacetic acid 95:5:0.1, v/v/v)			Mobile phase B (acetonitrile: water: trifluoroacetic acid 95:5:0.1, v/v/v)					

Resolution critical for the ideal separation of the compounds in the multicomponent analysis [41–45] is also tested for varying column temperatures, trifluoroacetic acid ratio in the mobile phase, and flow rate of the mobile phase. Mobile phase gradient is applied according to method 3 and method 4 (Table 1). There was not a significant difference in the resolution of the phenolic compounds when the chromatograms were taken at different temperatures 30 °C, 35 °C, and 40°C (trifluoroacetic acid ratio 0.1 and 0.5-0.7 ml/min flow rate). All compounds demonstrated resolution > 1.5 except internal standard which is separated with resolution from 0.82 to 1.27 (Table 2), therefore, 30 °C column temperature is selected. Resolution for chlorogenic acid was < 1.5 for trifluoroacetic the acid ratio of 0.01 and > 1.5 for the trifluoroacetic acid ratio of 0.05 or 0.1, therefore we continued with the trifluoroacetic acid ratio 0.1. Flow rate of 0.5 ml/min and gradient flow rate changing between 0.5 – 0.7 ml/min (column temperature 30 °C and acid ratio of 0.1) were both resulted in resolutions values of >1.5 for all parameters except internal standard. In addition, the resolution of the internal standard was higher at a mobile phase flow rate of 0.5 ml/min compared to that of 0.5-0.7 ml/min. Since backpressure was too high causing system disruption at high flow rates, especially at high concentration values, method 3 which applies a flow rate of 0.5 ml/min, column temperature of 30 °C, and acid ratio of 0.1 was preferred.

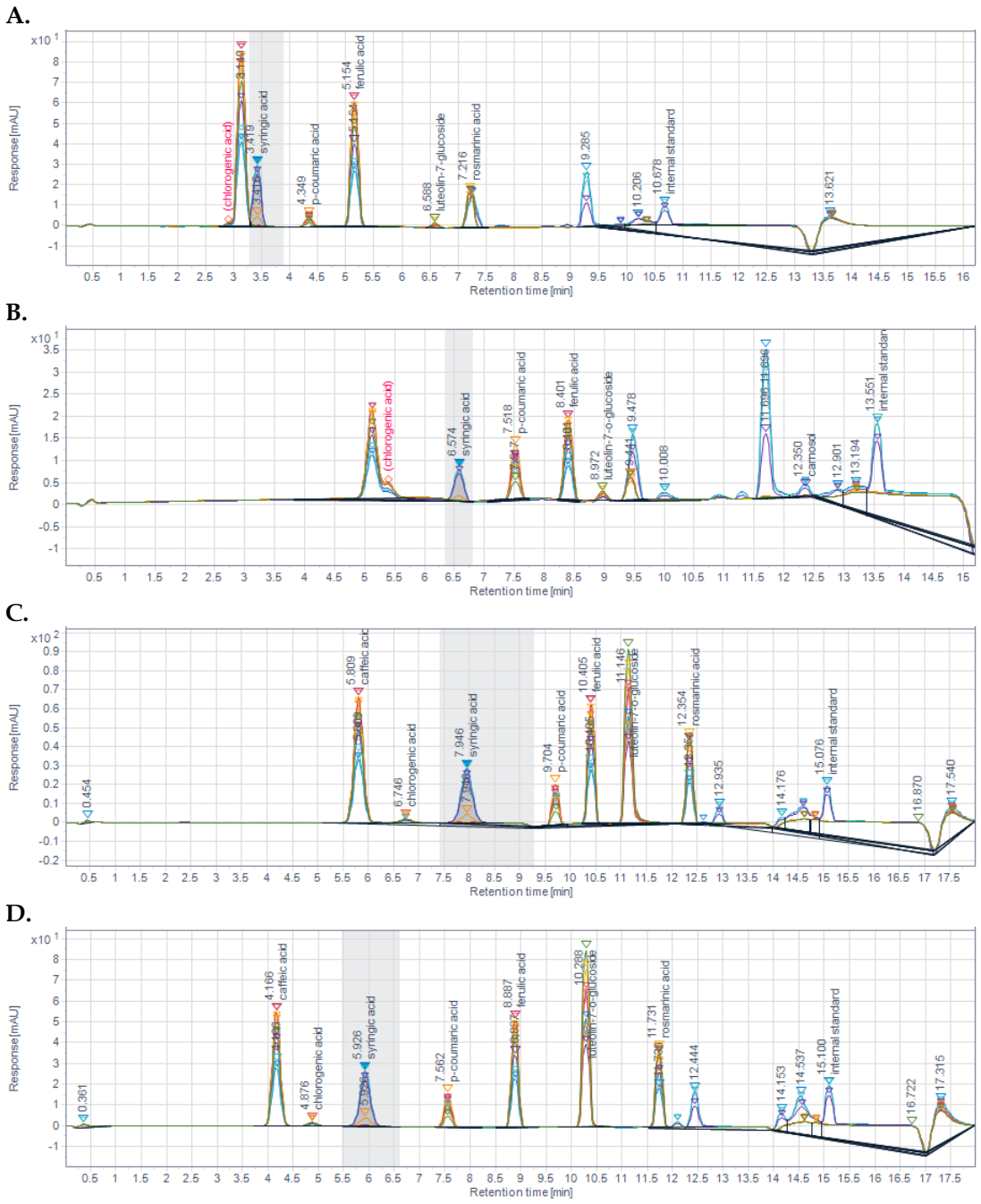

Figure 1. Chromatograms of mixture of pure compounds at 276, 278, 286, 300, 310, 322, 324 and 336 nm A) Method 1 B) Method 2 C) Method 3 and D) Method 4 given in Table 1.

All peaks are resolved, and the chromatographic run is completed in 18 minutes in method 3, in this study. In literature, varying types, and number of phenolic compounds from plant extracts were analyzed by HPLC using DAD or UV detectors demonstrated total run time of 45-97 minutes. In a study, 11 phenolic compounds including caffeic acid, ferulic acid, p-coumaric acid, rosmarinic acid and syringic acid were separated in 75 minutes [39]. Similarly, 10 phenolic compounds including caffeic acid, p-coumaric acid, rosmarinic acid and syringic acid were analyzed in 60 minutes [34]. In another study, 10 other phenolic compounds including caffeic acid, chlorogenic acid, ferulic acid and rosmarinic acid were separated in 65 minutes [19]. Caffeic acid, ferulic acid, p-coumaric acid, rosmarinic acid and one more phenolic compound were even separated in 97 minutes according to another study [7]. It has been also demonstrated that 3 phenolic compounds namely caffeic, ferulic and rosmarinic were separated in 45 minutes. The main drawback of these methods is that the relatively long run time varied by the type and number of phenolic

compounds separated. This could be attributed to the application of HPLC using columns with larger dimensions between 100- and 250-mm length and 4.6 mm internal diameter and particle size such as 5 µm. Shorter run times are reported only in a few studies that utilized the columns with smaller particle sizes. For example, rosmarinic acid along with 6 other phenolic compounds were separated in 5 or 12 minutes [16]. In addition, separation of 20 phenolic compounds including chlorogenic acid, caffeic acid, syringic acid, ferulic acid, p-coumaric acid and rosmarinic acid were achieved in 28 minutes [18]. Compound specific wavelengths were applied for separation of 7 phenolic compounds in this study. In the literature, methods typically employed either a single wavelength or a few wavelengths for detecting multiple phenolic compounds. The most common wavelengths used for detection were 278 nm [17], 280 nm [8,10,18,19,23,34] 288 nm [46] or 234 nm [18]. In some studies, multiple wavelengths were utilized, such as 278 nm, 340 nm [15], 280, 320 nm [11] 336 nm, 328 nm and 348 nm[5]. In limited number of studies, signal specific wavelengths are used for the quantifications [7,9,16,39].

Table 2. Resolutions of phenolic compounds at different column temperatures, flow rate of mobile phase and acid ratio of mobile phase A and B.

parameters	**conditions**					
Column (˚C)	**30**	**30**	**30**	**35**	**40**	**30**
Trifluoroacetic acid ratio[a]	**0.01**	**0.05**	**0.1**	**0.1**	**0.1**	**0.1**
Flow (ml/min)	**0.5-0.7**	**0.5-0.7**	**0.5-0.7**	**0.5-0.7**	**0.5-0.7**	**0.5**
phenolic compounds	**resolutions**					
Chlorogenic acid	0.83	1.91	2.07	2.11	1.86	2.62
Syringic acid	4.78	5.17	4.89	2.76	2.99	2.84
p-Coumaric acid	4.90	4.39	5.20	5.23	4.38	4.99
Ferulic acid	16.83	16.59	17.65	18.20	19.26	15.15
Luteolin-7-o-glucoside	5.47	5.60	5.74	5.76	6.22	2.97
Rosmarinic acid	4.75	5.33	5.67	5.54	5.28	4.60
Internal standard	0.99	1.04	0.87	0.91	0.82	1.27

[a] Trifluoroacetic acid ratio of 0.01 corresponds to mobile phase A (water: trifluoroacetic acid, 100:0.01, v/v) and mobile phase B (acetonitrile: water: trifluoroacetic acid, 95:5:0.01, v/v/v). Trifluoroacetic acid ratio of 0.05 corresponds to mobile phase A (water: trifluoroacetic acid,100:0.05, v/v) and mobile phase B (acetonitrile: water: trifluoroacetic acid, 95:5:0.05, v/v/v). Trifluoroacetic acid ratio of 0.1 corresponds to mobile phase A (water: trifluoroacetic acid,100:0.1,v/v) and mobile phase B (acetonitrile: water: trifluoroacetic acid, 95:5:0.1, v/v/v).

2.2. Extraction

The results of the extractions using different solvent volumes, composition and extraction duration for the capsules and powder show that the highest peak area was generated when 25 mg sample is dissolved in minimum amount of solvent, 1 ml. The total peak areas of the phenolic compounds increased by increasing sample to solvent ratio. Extraction of phenolic compounds from sage was achieved using hydrophilic solvents methanol or aqueous methanol according to literature[5,6,9,10,12,13,16,18,19]. Therefore, solvent and solvent mixtures of methanol, water and ethanol were tested for extraction of selected phenolic compounds from dietary sage capsules. Significant differences were not recorded for different compositions of ethanol, methanol and water. Therefore, the solvent with a composition of methanol: water (60:40, v/v) was used. Duration of extraction and extraction procedures of orbital shaking and ultrasonic bath were also evaluated. Slight improvement was observed when the extraction duration is increased to 120 minutes or ultrasonic bath was used, therefore extraction duration of 30 minutes using shaker is preferred with very small compensation from amount of analyte recovered. The best sample to solvent ratio was determined as 25 mg by dissolving in 1 ml solvent. Further experiments were performed by extracting 50 mg in 2 ml solvent volume which generates extract suitable to sample vials. As a result, optimized extraction procedure targets highest recovery of the phenolic compounds and practical with respect to solvent volume and steps aiming minimum waste. The optimized extraction procedure is applied throughout the studies of method validation.

2.3. System Suitability and Method Validation

System suitability and validation of method 3 are done. Capacity factor ≥ 2, tailing ≤ 2, number of theoretical plates ≥ 2000 and relative standard deviation (RSD%) for the retention time ≤ 5 % are the acceptance criteria. The number of theoretical plates of first eluting peak caffeic acid and last eluting peak rosmarinic acid were determined to be 4439 and 39278, respectively and peaks of other compounds have a number of theoretical plates within these values. RSD% in retention time, tailing, and asymmetry of all compounds were ≤ 0.09%, (0.99 - 1.06), and (0.99 - 1.10), respectively indicating the suitability of the method. Multiple guidelines, applicable to the analysis of plant extracts, are taken into consideration for method validation.

2.3.1. Selectivity

Validation for selectivity is done by comparison of chromatograms of the sample with that of blank matrix. Extracts of other plants were used as blank matrix [42,47] since it is difficult to obtain blank plant extracts lacking target compounds. For example, alfalfa powder is used for the analysis of Echinacea samples [27]. In this study, selectivity experiments are done by evaluation of capsule samples spiked with pure standards for each of the compounds, because targeted compounds may not be sufficiently present in actual samples [45,48]. The chromatogram is clear at the retention time of caffeic acid, chlorogenic acid and syringic acid (< 9 minutes). Some peaks are detected around p-coumaric acid, ferulic acid and rosmarinic acid (9- 15 minutes) but they did not prevent accurate identification and quantification (Figure 2A-F). Similar observations are obtained for powder samples (Figure 3A-F).

2.3.2. LOD and LOQ

There are several approaches for determination of LOD and LOQ according to method validation guidelines. USP suggest that LOD and LOQ corresponds the concentrations when S/N= 3 and 10, respectively, which is claimed to be insufficient (Betz et al. 2011) Indeed, we have obtained very low values of LOD and LOQ by applying USP method. AOAC suggests that LOD = x + 3std, LOQ = x + 10std (x: mean, std: standard deviation) based on the measurements (n=7) of the blank sample, which is not applicable to dietary sage capsules or powder because blank sample is not present. LOD = 3.3 std/a and LOQ = 10 std/a, (a: the slope and std is the standard deviation of the y-intercept) based on calibration curve measurements (n=6) according to the guidelines of the International Conference on Harmonization(ICH). Finally, according to the guidelines of the Environmental Protection Agency (EPA), LOD and LOQ corresponds to 3 std and 10 std, respectively where std is the standard deviation of the lowest level calibration solution by 7 measurements [44]. Here in, we have adopted the EPA approach for the determination of LOD and LOQ. Results are listed in Table 3 where, LOD and LOQ values of all phenolic compounds were 2.03 – 18.03 mg/ml and 6.77 – 60.10 mg/ml, respectively.

2.3.3. Linearity

The concentration range of all phenolic compounds is determined based on the signal peak areas of extracted samples run and the maximum absorptivity of each phenolic compound. The repeatability of the linearity has been confirmed by the calibration curves obtained at different days with fresh solutions, intra-assay and inter-assay injections of the calibration curves. Equations of the calibration curve and r^2 values are given in Table 3. Concentrations of the calibration solutions are prone to human error and expensive pure standards are limited, therefore, calibration solutions are not always prepared, instead solutions which are prepared and kept at -20 ˚C are used.

Calibration by addition of butyl hydroxy toluene as internal standard was examined as well, however, r^2 values were lower which is attributed to the late elution of internal standard with resolution < 1.5. Therefore, calibration with internal addition is not preferred for quantitation.

2.3.4. Accuracy & Precision

Relative error (RE%) and RSD% values assessing the accuracy and precision respectively are given in Table 4. Intra- assay and inter-assay RSD% were in the ranges of 0.17 - 10.80 and 0.84 - 8.31, respectively. Intra-assay and inter-assay RE% are in the ranges of (-13.63) - 4.95 and (-7.81) - 6.60, respectively, except luteolin-7-o-glucoside, which demonstrated the RE% > 15% for low and medium levels, but < 15% for high concentration levels.

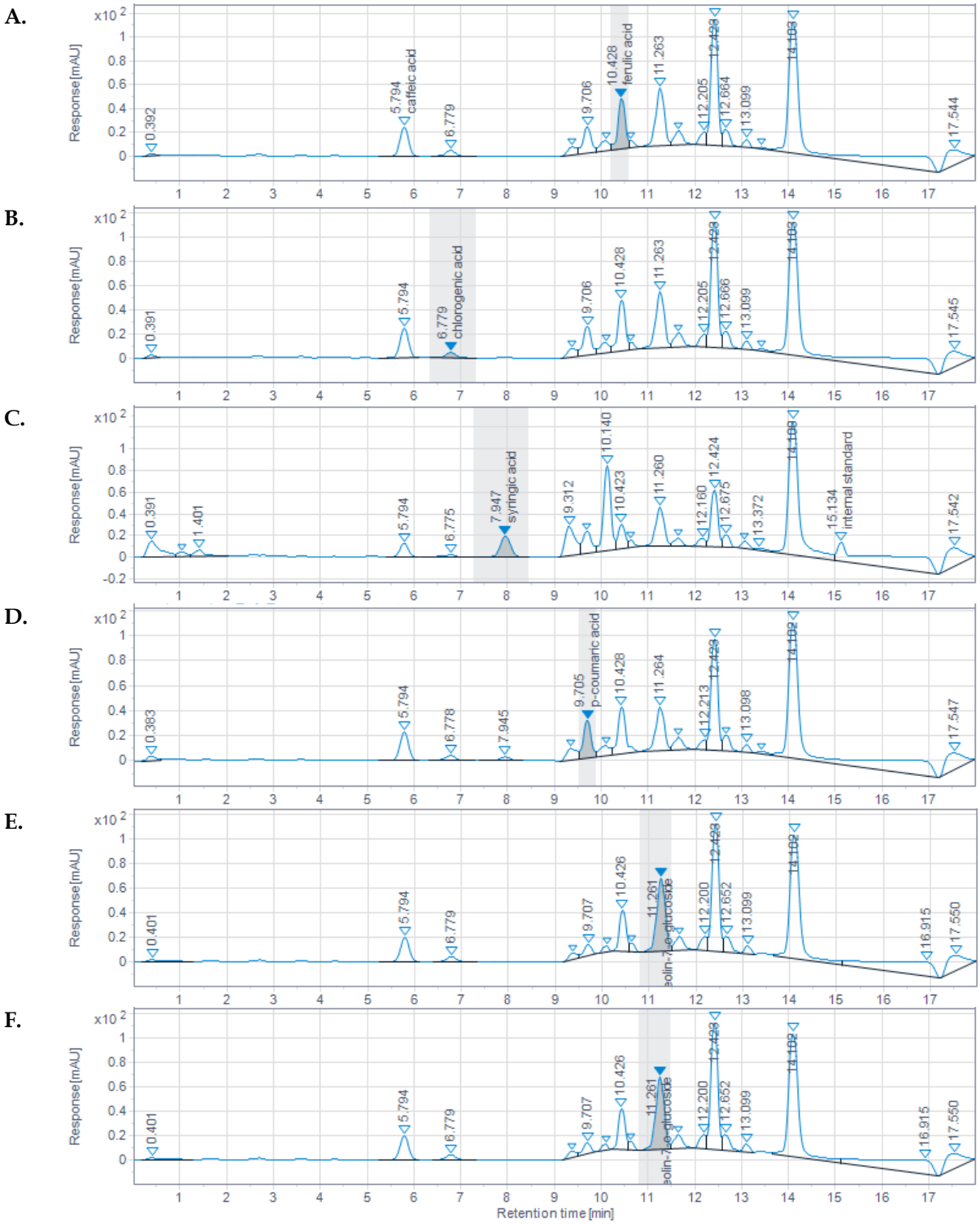

Figure 2. The extracted chromatograms of the capsule sample spiked with standard mixture of phenolic compounds at A)324 nm, B)322 nm, C)276 nm, D)310 nm, E)336 nm, F)330 nm corresponding to the chromatograms at maximum absorbances of caffeic acid/ferulic acid, chlorogenic acid, syringic acid, p-coumaric acid, luteolin-7-o-glucoside and rosmarinic acid, respectively.

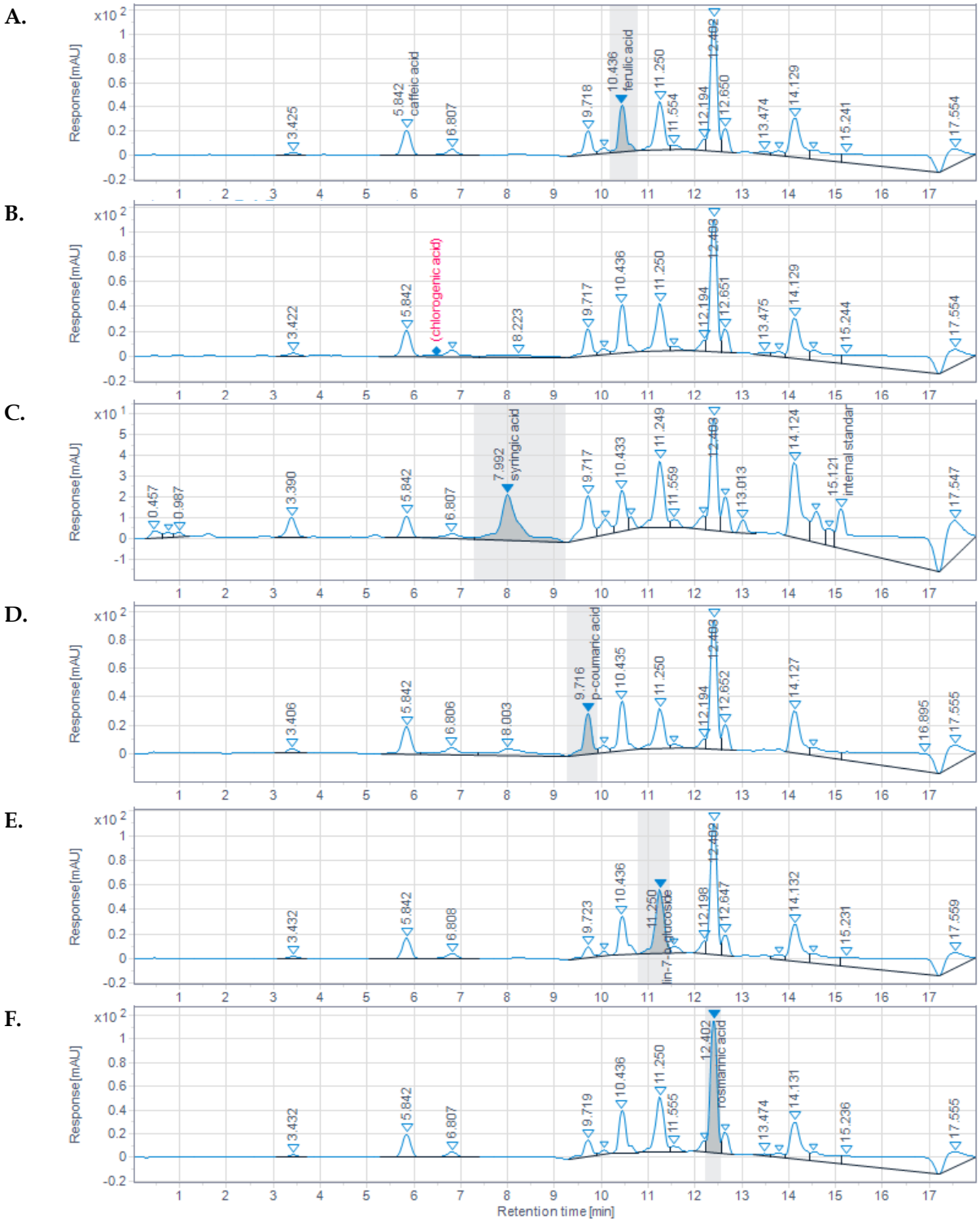

Figure 3. The extracted chromatograms of the powder sample spiked with standard mixture of phenolic compounds at A) 324 nm B)322 nm C)276 nm D) 310 nm E) 336 nm F) 330 nm corresponding to the chromatograms at the maximum absorbances of caffeic acid/ferulic acid, chlorogenic acid, syringic acid, p-coumaric acid, luteolin-7-o-glucoside and rosmarinic acid respectively.

2.3.5. *Recovery*

There are various approaches for validation of recovery experiments according to method validation guidelines which usually necessitate the blank matrix. Blank samples for plant extracts are difficult to obtain in contrast to human or animal biological samples since the composition of plants is more specific and variant. In only a few studies, extracts of other plants are used, and it is difficult to predict the secondary

metabolite composition of each plant. After careful investigation of the literature on plant extracts [27,41,44,45], in this study, recovery is evaluated by comparison of the measurements of the extracted sample (U), extracted sample spiked with pure standards of phenolic compounds(S), and pure standards (T) referring to sampling method. The appropriate number of pure standards are spiked on the sample and extractions are performed at three levels. Recovery% is calculated by (S -U)/T*100.

Recoveries for the dietary sage capsules capsule were between 91.0 and 113.0% except for ferulic acid and luteolin-7-o-glucoside (Table 4). Recovery for ferulic acid was 41.0% at low concentration levels and 76.0% at medium concentration levels indicating that as concentration increases, recoveries possibly improve. Therefore, a higher concentration range may be considered for ferulic acid. Unacceptable recovery for low concentration of luteolin-7-o-glucoside may be attributed to the difficulty in dissolving luteolin-7-o-glucoside, which was facilitated by the application of ultrasonic bath and heat. As a result, alternative procedures may be considered for dissolving luteolin-7-o-glucoside.

Table 3. LOD and LOQ values in mg/ml and linear regression equation and r^2 of calibration curve

Compounds	LOD	LOQ	linear regression equation	r^2
caffeic acid	1.97	6.58	10.6050x – 3.9275	0.9994
chlorogenic acid	5.69	18.97	5.7414x – 6.2008	0.9994
syringic acid	4.31	14.36	10.8918x – 4.9004	0.9995
p-coumaric acid	18.77	62.56	5.6661x – 3.9895	0.9993
luteolin-7-o-glucoside	3.58	11.95	6.2498x – 7.0167	0.9995
ferulic acid	2.68	8.95	16.1183x – 26.4477	0.9997
rosmarinic acid	6.26	20.87	6.1405x – 4.3687	0.9996

Recoveries for sage powder sample are also given in Table 4. Recoveries for some phenolic compounds, especially at low levels were not within the acceptable range of 85.0 – 115.0%. Recovery for caffeic acid at low concentration level is 77.0% which improves with increasing concentration. Recovery for ferulic acid 63.0% for medium concentration level. At low and medium levels of recovery of luteolin-7-o-glucoside have resulted in 58.0% and 75.0%, respectively. The recovery of rosmarinic acid is 134.0% at medium concentration level and are within range at medium and high concentration levels. Recoveries for rosmarinic acid, ferulic acid and syringic acid at low levels were inconsistent. Lower recoveries of the powder samples in comparison to capsule could be attributed to the complexity of the powder sample. Plant sources in capsule are subjected to extraction whereas powder samples are crude and possibly contain many interferent molecules requiring more elaborate sample preparation procedure before UHPLC analysis. A correction factor can be applied to measured concentrations at low levels to compensate the error due to low recovery of extraction.

Additionally, blank matrix is made up by dissolving subsidiary chemicals maltodextrin and magnesium stearate present in sage capsules [44]. Recoveries were from 85.0 to 106.0% when maltodextrin/magnesium stearate is used as blank matrix (Table 4). Higher observed recoveries for blank sample of maltodextrin/magnesium stearate compared to capsule indicates that interferences in the sample prevent recovery of target analytes.

2.4. Analysis of dietary sage capsules and powder

The analyses of three brands of sage capsule and powder obtained are done by the developed UHPLC method. The amounts of luteolin-7-o-glucoside and rosmarinic acid were highest in all capsule and powder samples with a significant variation among capsule samples. Determined values of rosmarinic acid concentration were 240.03 µg/ml, 135.11 µg/ml, and 80.04 µg/ml in brand 1, brand 2 and brand 3 capsules, respectively. that brand 1 has the highest concentration of both luteolin-7-o-glucoside and rosmarinic acid. In addition, Caffeic acid is only detected in brand 1 with a concentration of 10.43 µg/ml. These results demonstrate that brand 1 is the richest. Other phenolic compounds were below the quantitation limit. Concentrations of rosmarinic acid in powder samples were 246.17 µg/ml, which is comparable with brand 1 capsules sample. Also, significant amount of syringic acid (30.22 µg/ml) is observed in powder sample. As a result, variation in amounts of phenolic compounds in terms of rosmarinic acid and caffeic acid were observed in different brands of dietary supplements commercialized as sage capsules.

Table 4. Intra-assay and inter-assay accuracy, precision, and recoveries (%) of capsule samples, the powdered and blank solution made by adding magnesium stearate and maltodextrin.

		intra-assay		inter-assay		recovery%		
compounds	**spiked conc. (µg/ml)**	**RSD%**	**RE%**	**RSD%**	**RE%**	**capsule**	**powder**	**blank**
Caffeic acid	**5.80**	2.18	0.23	1.28	-2.65	114	77	85
	18.56	1.87	-7.74	8.25	2.45	99	87	94
	46.40	2.82	-12.94	6.97	-6.88	90	86	93
Chlorogenic acid	**17.90**	3.41	0.91	2.17	-0.99	96	93	87
	57.29	1.61	-7.46	8.31	2.13	97	89	91
	143.22	0.17	-13.63	6.68	-7.81	90	86	92
Syringic acid	**13.00**	3.47	4.95	1.98	2.16	102	-	94
	41.60	0.71	-0.34	5.58	6.60	99	101	100
	104.00	1.17	-4.72	2.72	-1.91	91	95	100
p-Coumaric acid	**40.00**	4.17	2.57	2.25	-0.49	97	102	92
	128.00	1.97	-3.27	7.14	4.81	98	96	96
	320.00	0.39	-7.97	4.85	-4.12	93	92	96
Ferulic acid	**8.20**	3.30	1.56	0.84	-0.90	41	-	90
	26.24	1.27	-5.37	7.88	3.73	76	63	95
	65.60	0.65	-10.23	5.99	-5.43	81	80	95
Luteolin-7-o-	**10.63**	10.28	26.80	-	-	-	58	92
glucoside	**34.00**	10.80	21.94	-	-	119	99	106
	85.00	7.42	6.40	-	-	102	75	100
Rosmarinic acid	**20.00**	4.47	3.74	2.18	0.81	106	-	92
	64.00	2.20	-3.35	7.52	5.17	102	134	96
	160.00	0.70	-8.55	5.11	-4.29	94	115	96

3. CONCLUSION

UHPLC-DAD method was developed and validated for quantification of seven phenolic compounds namely caffeic acid, chlorogenic acid, syringic acid, ferulic acid, p-coumaric acid, luteolin-7-o-glucoside and rosmarinic acid in dietary supplements of sage capsules and powder. Chromatographic conditions, especially mobile phase gradient were optimized for separation of the compounds with very similar features. The total run time of the analysis was 18 minutes and chromatographic suitability parameters were within the acceptable values. Sample preparation stages were also optimized for using less solvent and samples applicable in moderate laboratories. The selectivity of the method was improved by signal-specific integration and quantitation of the phenolic compounds with the use of concurrent DAD detectors available in quality control laboratories. Method validation procedures, especially for selectivity and recovery suitable for capsules and powder samples of plant extracts are proposed. As a conclusion, the method is offered for quality control of sage capsules by quantitation of seven phenolic compounds with a short time and simple sample preparation stage. The method could also be applied for the analysis of identical phenolic compounds in dietary supplements of extracts obtained from similar plants and herbs. This study contributes to the literature by developing a UHPLC-DAD method that significantly reduces analysis times and enhances selectivity, addressing common limitations of traditional HPLC methods used for phenolic compound analysis. By applying signal-specific integration with DAD detectors, which has been underexplored, this method offers a cost-effective alternative to the more expensive LC-MS/MS for quality control laboratories. Furthermore, it fills a gap in the literature by focusing on the quantitation of phenolic compounds in commercial dietary sage capsules, an area that has been minimally covered.

4. MATERIALS AND METHODS

4.1. Materials

Pure standards of chlorogenic acid, ferulic acid, p-coumaric acid, carnosol, luteolin-7-o-glucoside, syringic acid, rosmarinic acid, and butylhydroxytoluene were purchased from Sigma-Aldrich. Magnesium stearate and maltodextrin were obtained from Sigma-Aldrich (St.Louis, MO, USA). HPLC grade methanol, trifluoroacetic acid, acetonitrile and ethanol were purchased from Isolab Laborgerate GmbH (Eschau, Germany). Disposable polytetrafluoroethylene membrane filters with 0.45 µm pore size and 13 mm diameter was obtained from Macherey Nagel (Duren, Germany). Water ultra purification system (Sartorius Arium Comfort, Goettingen, Germany) was used for water supply for mobile phase and sample preparation.

Sage powder and 3 different brands of supplements of sage capsules (brand 1, brand 2 and brand 3) were obtained from various web-based suppliers. The composition of the products on their label are given as extracts of sage, *plantago media*, oregano, St. John's Wort, cordyceps and grape for brand 1, *salvia*, bitter melon, myrtle and senna for brand 2, and extracts of sage (*salvia officinalis*), fumeroot (*fumaria officinalis*) and lady's mantle (*alchemilla vulgaris*) in brand 3. Sage powder is described as sage extract on the product label.

4.2. Extraction of sage capsules and powder

Capsule samples were extracted according to procedures in literature (Brown et al. 2008).Homogenous mixture was obtained by mixing 10 capsules. Then, the appropriate amount of sample was transferred to the centrifugation tubes and solvent is added and dissolved using rotational mixer for 30 minutes. The ratio of organic solvent to water, type of extraction solvent, volume of the solvent, duration of extraction was modified. Powder samples were also prepared in the same manner.

Solvent volumes examined ranged between 1 - 5 ml. Solvent compositions tested were methanol: water (90:10, 70:30, 60:40, 40:60, 30:70, ethanol: water (40:60, v/v), methanol: water: ethanol (30:40:40 and 40:20:40, v/v). Duration of the orbital shaker and ultrasonic bath were 30 min, 60 min and 120 min. Supernatant was separated by centrifugation at 4000 rpm for 5 minutes and filtered using the filter membrane before UHPLC analysis.

4.3. UHPLC Analysis

UHPLC analyses were performed using the instrument composed of UHPLC-DAD system (Agilent Technologies, Santa Clara, CA, USA). UV/VIS (Ultraviolet-Visible) spectrum of the compounds are taken on DAD detector of HPLC using acetonitrile: water. Data acquisition and processing are done using Agilent Open Lab CDS software. Separations of the compounds were achieved in reverse mode using the C18 column. As a mobile phase aqueous acetonitrile is used. Other conditions determined are presented in the results and discussion.

4.4. Preparation of Standard Stock Solutions and Calibration Solutions

Standards of the phenolic compounds are stored at room temperature, -4 °C and -20 °C. Methanol: water (6:4, v/v), ethanol: water (1:1, v/v) or ethanol were used as the solvent to prepare the stock solution of phenolic compounds. Ethanol is used as the solvent for the preparation of butylhydroxytoluene (BHT), which is an internal standard. Appropriate amounts of stock solution of phenolic compounds were mixed for obtaining mixture solution, which was diluted to make calibration solutions at 6 concentration levels. Methanol: water (v/v, 60:40) was used as the solvent and each calibration solution is spiked with internal standard. Concentration calibration solutions were 9.28 to 37.12 mg/ml for caffeic acid, (28.64 - 114.58) mg/ml for chlorogenic acid, (13.12 - 52.48) mg/ml for ferulic acid, (17.00 - 68.00) mg/ml for luteolin-7-o-glucoside, (64.00 – 256.00) mg/ml for p-coumaric acid, (32.00 – 128.00) mg/ml for rosmarinic acid, (20.80 - 83.20) mg/ml for syringic acid. All stock solutions were stored in small proportions at -80 °C until use for long term. Stock solutions are stored at - 20 °C for short term. All solutions are stored away from light.

4.5. System Suitability Tests and Method Validation

Gradient program is optimized. Resolution of the phenolic compounds were determined for column temperatures (30 °C, 40 °C, 45 °C), and flow rate of the mobile phase (0.5 - 0.7 ml/min) and acid addition to 100 ml of mobile phase in volume ratios of 0.01, 0.05, and 0.1. System suitability parameters, theoretical plate number, capacity factor, tailing and asymmetry factor were also determined. Calculations were done according to the United States Pharmacopeia (USP) guidelines. Method validation was done according to guidelines of the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) and

Association of Analytical Chemist (AOAC). The selectivity of the method was evaluated by comparing the chromatograms of extracted sample, extracted sample spiked with pure standard solutions and pure standard solutions. Linearity was tested by running 6 calibration solutions on different days (n=3). Agilent Open Lab Software program was used for the generation of the calibration curve which was expressed with an equation y = ax + b, where y: peak area, x: concentration, a: slope, and b: intersection on the y-axis. r2 values were also determined. Calibration by external standard and internal standard additions were both evaluated. The LOD and LOQ)were determined according to EPA guidelines by measuring lowest concentration of calibration solution 7 times. Method validation for precision and accuracy (intra-assay and inter-assay) were assessed by measuring the concentrations of phenolic compounds at 3 levels (n = 3). Relative error (%RE) and relative standard deviation (%RSD) for assessing accuracy and precision were calculated according to the equations, %RE = {(theoretical concentration–measured concentration)/theoretical concentration}×100. %RSD={standard deviation of measured concentration/mean of the measured concentration} x 100. Method validation for recovery was performed for capsule and powder samples separately. In addition, magnesium stearate and maltodextrin mixture representing capsule matrix were also used for testing recovery. Extracted sample, extracted sample spiked with pure standards and pure standards were measured to calculate recovery %. Throughout all experiments, optimized extraction procedure is applied as explained in results and discussion.

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