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GS-MS based metabolite profile of different trademarks of fenugreek

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ABSTRACT

GS-MS based metabolite profile of fenugreek (*Trigonella foenum-graecum* L.) of three different trademarks was investigated. In polar fractions totally 40 primary metabolites (carbohydrates, organic and amino acids) and 9 phenolic acids (with caffeic and 4-hydroxybenzoic acids as major) were identified. The dominant metabolites in non-polar fraction were palmitic, linoleic, oleic and stearic acids. Two untypical triterpenic acids (oleanolic and ursolic acid) for family Fabaceae were identified in one of the analyzed sample. The presence of these triterpenoids in the sample of fenugreek indicates for probably adulteration, during the production process, with spices from other families, such as Lamiaceae or Rosaceae. The data of provided GC-MS analyses and subsequent principal component analysis (PCA) and hierarchical cluster analysis (HCA) revealed that investigated samples of the same species from three different trademarks had significant differences in their phytochemical content. Thus could be base for developing a protocol for quality control based on metabolomics approach.

Key words: Fenugreek, GC-MS, PCA, HCA

Introduction

Spices have been used from ancient time to impart aroma, color and taste of food products. In addition, they have wide application in modern food production, because of their valuable biological activities, such as antimicrobial and antioxidant properties (Heperkan, 2006; Parthasarathy et al., 2008).

One of the wide spread spices is fenugreek (*Trigonella foenum-graecum* L.). It is an aromatic herb belonging to the family Fabaceae that grows spontaneously in large areas of Europe and Asia (Dinu et al., 2013). It has been used as a culinary spice, a flavoring agent and as a medicinal plant from ancient time (Ghosh et al., 2015). In modern food practice, the seeds or the extract are used in bakery products, frozen dairy products, meat products, relish, condiments, candy, gravy sauces, and gelatin puddings and in alcoholic and non-alcoholic beverages (Moradi kor et al, 2013). In Bulgarian culinary fenugreek is used individually or as mixture with other spices in production of meat products, mainly because of its antiseptic properties. Besides, fenugreek is the main compound of the traditional Bulgarian spice "sharena sol" (Atanasova, E., 2011).

Besides in culinary, seeds and leaves of the fenugreek were used in folk medicine for the treatment of wounds, abscesses, arthritis, bronchitis, ulcer, digestive and kidney problems, temporary loss of appetite, inflammations of the skin, to reduce blood glucose levels and bad cholesterol in human body. Fenugreek has also been reported to exhibit antitumor, antiviral, antimicrobial, anticancer and antioxidant properties (Toppo et al., 2009; Patel and Dhanabal, 2013, Moradi kor et al.; 2013; Sheikhlar, 2013). These biological and pharmacological activities were associated with the presence of various bioactive constituents, such as alkaloids,

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flavonoids, steroids, saponins, vitamins and amino acids (Moradi kor, 2013; Ghosh et al., 2015). Identifying of these compounds is a purpose of metabolite analysis. Nowadays, metabolomics is a powerful tool for quality control and food safety (Cevallos-Cevallos et al., 2013). One of the most useful and reliable method for metabolite analyses of complex biological matrixes, such as plant samples is gas chromatography-mass spectrometry (GC-MS) (Kopka J. 2006).

Thus the purpose of current study was GC-MS metabolite profiling of commercially available products of *T. foenum-graecum* and subsequent statistical analyses of obtained data.

Materials and methods

Plant material

Samples of fenugreek were bought from three different randomly chosen companies, offer spices in the Bulgarian markets.

Samples preparation

Samples of 50 mg of dried drug were extracted with 1.0 mL methanol for 30 min at 70 ºC in termomixer (950 rpm). After centrifugation (1 min, 5000 rpm), 500 µL of supernatant was mixed with 500 µL chloroform and 500 µL d H_2O. Samples were vortexed, then centrifugated (1 min, 5000 rpm) and polar fraction was divided in two sub-fractions (polar fraction I and II).

Polar fraction I was dried under vacuum (30 ºC, 90 min). After addition of 50 µL pyridine (Sigma) and 50 µL metoxyamine hydrochloride (Sigma), samples were shaken and incubated in termomixer (1 h, 80ºC). After reaction time, 50 µL of N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA, Sigma) was added and samples were shaken in termomixer (1 h, 80ºC).

After adding the 0.5 mL of 1N NaOH to polar fraction II , samples were incubating for 16 hours in dark, then acidified with HCl (pH 1-2) and shaken for 60 min at 90 ºC in termomixer. After the reaction time the samples were extracted with ethyl acetate (3x500 µL) and the combined ethyl acetate fractions were dried over anhydrous sodium sulfate and evaporated to dryness under vacuum at 30ºC. After that, samples were dissolved in 50 µL pyridine (Sigma) and 50 µL N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA, Sigma) and were shaken in termomixer (1 h, 80ºC).

To non-polar fractions 500 µL of 2 % H_2SO_4 in methanol were added. After incubating in termomixer (1 h, 90ºC), samples were cooled and extracted with n-hexane (3x500 µL). The combined hexane extracts were dried over anhydrous sodium sulfate and evaporated to dryness under vacuum at 30 ºC. After that, samples were dissolved in 50 µL pyridine (Sigma) and 50 µL N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA, Sigma) and were shaken in termomixer (1 h, 80ºC). The obtained polar fractions I, II and non-polar fraction were used for GC-MS analysis.

GC-MS analysis

GC-MS analysis were carried out on gas chromatograph Agilent Technology Hewlett Packard 7890 A, coupled with mass detector Agilent Technology 5975 C inert XL EI/CI MSD at 70 eV). Separation of metabolites was on HP-5MS column (30 m x 0.25 mm x 0.25 µm) at temperature program : from 100°C to 180°C with the step of 15 °C/min and from 180°C to 300°C with the step of 5°C/min then hold on 300°C for 10 min. The injector temperature was 250 °C and the flow rate of carrier gas (helium) of 1.0 mL/min was used. The injection volume was 1µL.

Identification of the metabolites

The obtained mass spectra were read using 2.64 AMDIS (Automated Mass Spectral Deconvolution and Identification System, National Institute of Standardization and Technology (NIST), Gaithersburg, MD, USA). The separated polar and non-polar compounds were identified by comparison of their GC-MS spectra and Kovach retention index (RI) with referent compounds in NIST 08 database (NIST Mass Spectral Database, PC-Version 5.0, 2008). The RIs of compounds were recorded with standard n-hydrocarbon calibration mixture (C10-C40, Fluka) using 2.64 AMDIS software.

Data analyses

All analyses were performed in triplicate and data were expressed by means. In order to determine the most variable metabolites among the studied samples principal component analysis (PCA) was performed. To reveal sample relationships hierarchical cluster analysis was applied. On-line platform MetaboAnalyst 2.0 was used for statistical analysis.

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Results

In polar fraction I of analyzed samples of fenugreek 40 primary metabolites were identified, namely 13 carbohydrates, 15 organic acids, 10 free amino acids and urea and phosphoric acid (Table 1). The results are presented as % of Total Ion Current (TIC).

In non-polar fraction 6 saturated (caprylic, lauric, myristic, pentadecanoic, palmitic and stearic acid) and 2 unsaturated acids (linoleic and oleic acids) were identified. The investigated samples contain also glycerol, phythol, 1-monohexadecanoilglycerol, octadecanol, hexacosanol, octacosanol, α-tocopherol, campesterol, stigmasterol and β-sitosterol (Table 2).

GC-MS analysis of polar fraction II revealed the presence of 9 phenolic acids, divided in two structural types: hydroxybenzoic (4-hydroxybenzoic, salycilic, syringic, gentisic and protochateuic) and hydroxycinammic acids (ferulic, caffeic and p-cummaric) (Table 3).

Based on the obtained GC-MS results of three investigated samples of fenugreek Principal Component analysis (PCA) was performed. PCA biplots of polar fraction I, non-polar fraction and polar fraction II were presented in figure 1, figure 2 and figure 3, respectively.

Discussion

GC-MS analysis of polar fraction-I showed quality and quantity differences in the primary metabolites in the three investigated samples of fenugreek. Glucose was not found in sample 1, as iso-glucose was identified only in sample 2. Maltose and threhalose were absent in sample 3 (Table 1). Malonic and 3,4-dixydroxybutanic acids were not found in sample 3. Glucaric acid was synthesized only in sample 2. Amino acid composition of Sample 2 differ considerably from those of other two samples.

Concerning the identified metabolites in non-polar fraction of the analyzed samples of fenugreek interesting distinguishes also have been found (Table 2). Two uncommon compounds for family Fabaceae were identified in sample 1, namely oleanolic and ursolic acid. The presence of these triterpenoids in the sample of fenugreek indicates for probably adulteration with spices from other families, such as Lamiaceae or Rosaceae.

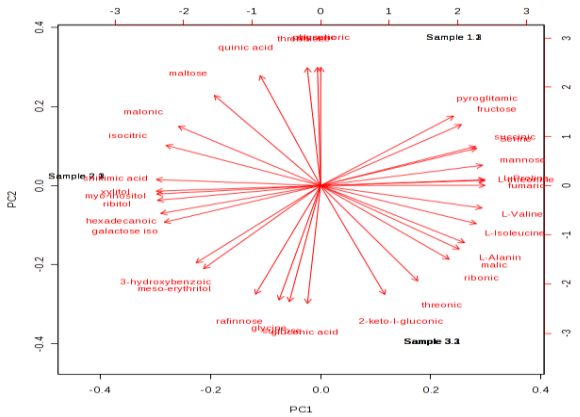

Figure 1. *PCA biplot of GC-MS data of polar fraction I of analyzed three samples of fenugreek.*

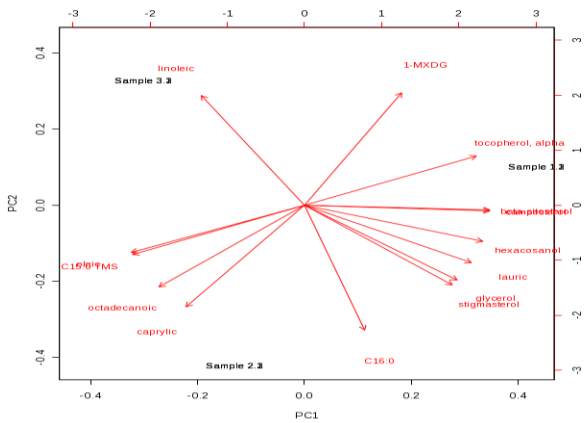

Figure 2. *PCA biplot of GC-MS data of non-polar fraction of analyzed three samples of fenugreek.*

In non-polar fraction valuable biologically active compounds were found, such as phytosterols, unsaturated fatty acids (linoleic and oleic), fatty alchohols and α-tocoferol. Phytosterols decrease levels of bad cholesterol in human body, as well as have anti-inflammatory and protective activity against external attacks (Bartnikowska, 2009).

The identified metabolites (carbohydrates, amino acids, organic acids and non-polar compounds) are products of the primary metabolism of plant cells. They are important for normal growth and cell division, as well as serve as initial compounds for synthesis of various bioactive secondary metabolites, such as polyphenols, which are from high importance for foods concerning their antioxidant capacity.

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Table1. *Chemical composition of polar fraction I of analyzed samples of fenugreek.*

Compound	Retention time (RT), min	TIC, % Sample 1	TIC, % Sample 2	TIC, % Sample 3
Carbohydrates				
Mezo-erythrol	7,64	0,53	1,58	1,23
Xylitol	9,80	1,12	1,31	1,13
Ribitol	10,11	2,72	29,13	4,19
Fructose	11,46	26,28	3,00	7,70
Glucose	11,52	6,06	nd	nd
Mannose	11,85	7,32	0,52	3,90
iso- Glucose	13,20	nd	17,98	nd
iso- Galactose	13,20	4,81	9,93	6,20
Myo-inosistol	15,67	0,46	1,00	0,51
Sucrose	22,79	10,28	18,78	27,29
Maltose	23,36	0,35	1,02	nd
Threhalose	24,52	16,87	0,96	nd
Raffinose	34,79	0,90	5,17	7,61
Organic acids				
Malonic acid	5,01	0,03	0,11	nd
Succinic acid	5,78	1,70	0,64	1,15
Glyceric acid	5,99	1,04	0,55	0,25
Fumaric acid	6,08	1,46	0,49	1,35
3,4-dihydroxy butanoic acid	6,91	0,01	0,01	nd
Malic acid	7,44	8,67	2,41	29,56
Threonic acid	7,99	0,41	0,28	2,63
2,3,4-trihydroxybutanoic acid	8,17	nd	0,08	nd
Shikimic acid	9,75	0,03	0,06	0,03
2-keto-l-gluconic acid	10,56	0,26	0,28	0,66
Ribonic acid	10,73	0,43	0,41	0,46
Glucaric acid	11,32	nd	0,11	nd
Iso-citric acid	11,39	0,02	0,10	nd
Quinic aicd	12,108	3,87	2,72	0,15
Gluconic acid	14,55	nd	0,04	0,14
Amino acids				
L-Alanin	3,70	0,27	0,15	0,40
L-Glycine	3,99	0,18	0,70	1,34
L-Valine	4,85	0,19	nd	0,32
Isoleucine	5,63	0,07	nd	0,15
L-Proline	5,67	2,05	nd	1,13
L-Serine	6,27	0,17	nd	0,06
L-Threonine	6,53	0,16	nd	0,12
Pyroglutamic acid	7,75	0,51	0,27	0,34
L-Glutamic acid	8,72	nd	0,12	nd
L-Phenylalanine	8,83	0,03	nd	nd
Others				
Urea	5,06	0,02	nd	nd
Phosphoric acid	5,47	0,70	0,10	nd

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Table 2. *Chemical composition of non-polar fraction of analyzed samples of fenugreek.*

Compound	Retention time (RT), min	TIC, % Sample 1	TIC, % Sample 2	TIC, % Sample 3
Caprylic acid	5,29	nd	0,03	0,01
Glycerol	5,42	1,85	1,37	0,50
Lauric acid	9,00	0,03	0,02	0,01
Phytol 1	11,36	0,64	nd	nd
Myristic acid	11,51	nd	0,11	0,11
Pentadecanoic acid	12,96	nd	0,08	0,05
Palmitic acid	14,46	28,15	36,48	19,29
Octadecanol	16,19	nd	0,04	nd
Linoleic acid	17,08	22,65	19,48	47,85
Oleic acid	17,17	8,27	26,06	21,88
Stearic acid	17,56	4,73	10,63	7,50
1-monohexadecanoilglycerol	23,01	0,21	nd	0,08
Hexacosanol	27,74	1,08	0,24	0,06
Octacosanol	30,25	0,28	nd	nd
α-tocopherol	30,37	0,30	nd	0,01
Campesterol	31,76	2,73	0,48	0,25
Stigmasterol	32,13	2,55	0,80	nd
β-sitosterol	32,8	19,75	4,18	2,39
Oleanolic acid	36,23	0,48	nd	nd
Ursolic acid	37,02	6,31	nd	nd

Table 3. *Chemical composition of polar fraction II of analyzed samples of fenugreek.*

Compound	Retention time (RT), min	TIC, % Sample 1	TIC, % Sample 2	TIC, % Sample 3
Salycilic acid	7,63	15,44	nd	22,56
4-hydoxybenzoic acid	8,78	15,76	2,04	42,48
Vanilic acid	10,48	6,69	nd	9,70
Gentisic acid	10,67	18,65	nd	15,17
Protocatechuic acid	11,26	3,12	nd	nd
Syringic acid	12,39	4,41	nd	nd
Ferulic acid	15,33	2,11	nd	nd
Caffeic acid	16,06	33,82	97,96	nd
p-cummaric acid	12,95	nd	nd	10,09

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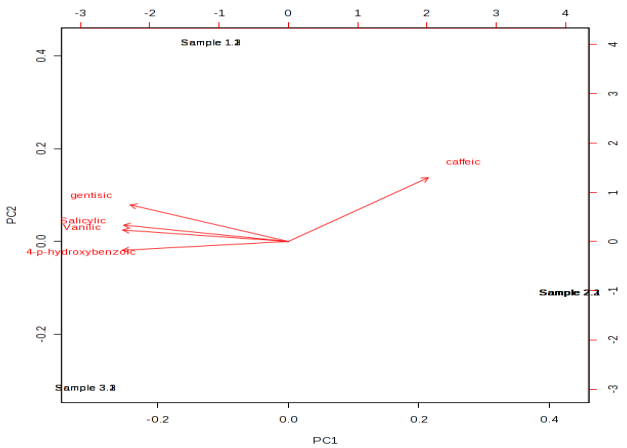

Figure 3. *PCA biplot of GC-MS data of polar fraction II of analyzed three samples of fenugreek.*

Significant differences in the phenolic acids in polar fraction II of analyzed samples was observed. Sample 2 contains only caffeic and p-hydroxybenzoic acid, as protocathehuic, syringic, ferulic and caffeic acid were not identified in sample 3.

In order to obtain clear overview of the variation of identified metabolites in the three samples Principal Component analysis (PCA) was performed.

PCA biplot of identified metabolites in polar fraction I demonstrated significant differences between the three samples. PC1 explained 61.6 % of the total variance in the data set while PC2 explained 38.4%. Sample 1 was located on the right half of plot because of its high content of piroglutamic acid, fructose, succinic acid, serine, proline, mannose, quinic and phosphoric acid. Sample 2 differ from others with higher contents of meso-erithrol, iso-galactose, ribitol, myoinositol, xylitol, shikimic acid, iso-citric and malonic acid and maltose. Sample 3 was located on the lower right half of the plot that could be explained with higher amount of valine, isoleucine, alanine, glycine, malic, ribonic, threonic, 2-keto-1-gluconic, gluconic acid and raffinose.

The analyzed three samples of fenugreek had significant statistical differences regarding the identified compounds in unipolar fractions (Figure 2). PC1 explained 66.8 % of the total variance in the data set while PC 2 explained 33.2 %

Sample 1 was in the right half of the plot near to PC2 zero point because of higher relative quantities of monohexadecanoilglycerol, α-tocoferol, β-sitosterol, hexacosanol, layrinic acid, glycerol and stigmasterol. Sample 2 and 3 were located in the opposite site of the plot as differentiated from others by palmitic, caprylic, stearic, pentadecanoic and oleic acid, as Sample 3 had highest content of linoleic acid (Figure 2).

Provided PCA of data of polar fraction 2 revealed that Sample 2 differ from the others with higher content of caffeic acid, as Sample 1 and 3 with higher amount of p-hydroxybenzoic, vanilic and salycilic acid.

The similarity between the investigated samples was defined by hierarchical cluster analysis (HCA), visualized by dendrograms. On the basis of metabolites in polar fraction 1 and 2, Sample 1 had higher similarity with sample 3 and formed 1 cluster (Figure 4 and 5). The observed lower distance between Sample 1 and 3 was due to the higher content of mannose, galactose, ribitol, xylitol, myo-inositol, fumaric, succinic, shikimic acid, prolin, serin, valine, isoleucine, alanine, in one hand and higher amount of phenolic acids, in other hand.

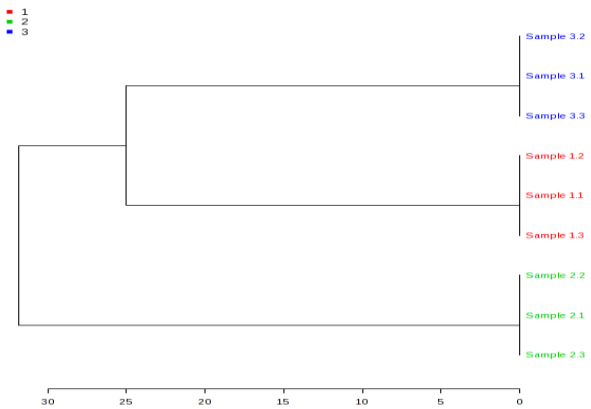

Figure 4. *Dendrogram of HCA of GC-MS data of polar fraction I of analyzed three samples of fenugreek.*

According to the obtained dendrogram of non-polar fractions, sample 2 was closer to the sample 3 and were grouped in one cluster (Figure 6). These samples contained higher amount of caprylic, octadecanoic, oleic and stearic acid.

The results of provided HCA corresponded with the data of PCA and could be used in classification of plant species and for quality and safety control of foods, as well.

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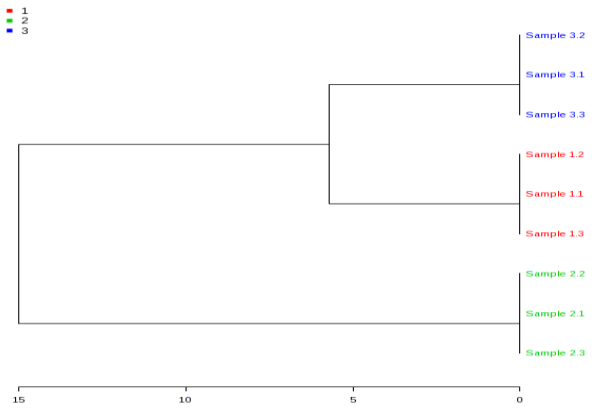

Figure 5. *Dendrogram of HCA of GC-MS data of polar fraction II of analyzed three samples of fenugreek.*

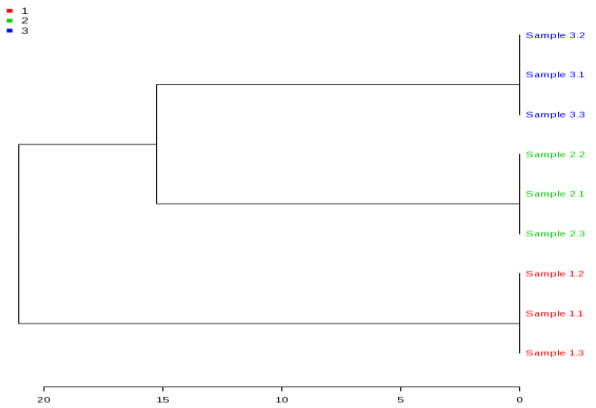

Figure 6. *Dendrogram of HCA of GC-MS data of non-polar fraction of analyzed three samples of fenugreek.*

Conclusion

The results of provided GC-MS analyses and subsequent PCA and HCA demonstrated that analyzed samples of the same species from three different trademarks had significant differences in their phytochemical content. This could be due to differences in climate and geographical conditions and time of harvest the crop, as well. However the presence of uncommon for family Fabaceae metabolites, oleanolic and ursolic acid is a clear sign that manufacturer of this sample had significant problem in production process and following the rule of GMP. The most probably reason for the attendance of these triterpenoids in the sample of fenugreek was contamination of product with plant drug of other genus, such as Lamiaceae. Results obtained clearly demonstrated that metabolomics approach is a power tool for control of quality and safety of food products.

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