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Quality-controlled LC-ESI-MS food metabolomics of fenugreek (*Trigonella foenum-graecum*) sprouts: Insights into changes in primary and specialized metabolites

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.) is an important food and spice with bioactive compounds against diabetes. In this study, fenugreek seeds germinating in darkness for 72 h were studied using quantification of trigonelline and 4-hydroxyisoleucine and an LC-ESI-MS/MS-based metabolomic approach capable of accurately estimating 237 features from various primary and specialized compound classes.

During germination, the concentrations of trigonelline and 4-hydroxyisoleucine rose by 33.5% and 33.3%, respectively. At the same time, untargeted metabolomics revealed 9 putative flavonoids increasing 1.19- to 2.77-fold compared to the dormant seeds. A set of 19 steroid saponins rose by 1.08- to 31.86-fold. Primary metabolites however showed much more variability: abundance changes in amino acid derivatives, peptides and saccharides fell in the 0.09- to 22.25-fold, 0.93- to 478.79-fold and 0.36- to 941.58-fold ranges, respectively.

To increase biosynthesis of specialized metabolites during germination, sprouts were exposed to 1–100 mM methyl jasmonate (MeJA) and methyl salicylate (MeSA). The hormone treatments affected normal metabolism: 67.1–83.1 % and 64.1–83.5 % of compounds showed a reduction compared to the controls in 100 mM MeJA and MeSA treatments at different sampling time points. Contrary to expectations, the abundance of flavonoids decreased, compared to the control sprouts (0.75- and 0.68-fold change medians, respectively). The same was observed for most, but not all steroid saponins.

The quality-controlled untargeted metabolomics approach proved to yield excellent insight into the metabolic changes during germination of fenugreek. The results suggest that although fenugreek germination causes major shifts in plant metabolism, there are no major qualitative changes in bioactive specialized metabolites during the first three days. This stability likely translates into good bioactivity that is similar to that of the seeds. Because the large changes in the primary metabolites likely alter the nutritive value of the seed, further studies are warranted.

1. Introduction

1.1. Fenugreek seeds and sprouts

Fenugreek (*Trigonella foenum-graecum* L., Fabaceae) has a long history of culinary uses and is cultivated in several countries of the world outside its original habitat. India is the largest producer of fenugreek, with an annual production of 45,000–55,000 tons (Yao et al., 2020). The plant is used in a variety of food products, for example curry, pickles, artificial maple syrup and vanilla extracts.

Abbreviations: DW, dry weight; ESI, electrospray ionization; FWHM, full width at half maximum; LC-ESI-MS, liquid chromatography - electrospray ionization - mass spectrometry; MeJA, methyl jasmonate; MeSA, methyl salicylate; QC, quality control.

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Fenugreek is also one of the well-researched medicinal plants that is consumed as a functional food against impaired glycemic control, metabolic disorders and obesity (Avalos-Soriano, De la Cruz-Cordero, Rosado, & Garcia-Gasca, 2016; Hamden et al., 2010; L. Liu, Du, Zhang, & Zhou, 2018; Mayakrishnan et al., 2015; Verma et al., 2016). This application is supported by a *meta*-analysis based on 12 controlled clinical trials, as recently concluded by Khodamoradi et al. (2020). A list of products currently available on the global market has recently been compiled as well (Yao et al., 2020). These include products for insufficient lactation, products to improve glycemic control and agents against respiratory tract diseases, mostly in combination with other herbs.

Fenugreek's bioactive compounds (Fig. 1) are biosynthesized through a variety of biosynthetic routes. The most abundant bioactive constituent is a galactomannan polysaccharide which accounts for 25–45 % of the dry weight (DW) of the seeds (Y. Liu et al., 2020). Small-molecule bioactives include the non-proteinogenic amino acid 4-hydroxyisoleucine (0.015–0.4 % DW) (Avalos-Soriano et al., 2016), the alkaloid trigonelline (0.26–0.39 % DW) (Laila et al., 2019), and the steroidal saponin trigofoenosides and trigoneosides, which are furostanol saponins biosynthesized by glycosylation of proto-diosgenin, proto-yamogenin, proto-gitogenin, proto-neogitogenin or similar aglyca. The glycosyl moieties of saponins consist of possibly branched-chain oligosaccharides of 2–6 monomers of glucose, xylose and rhamnose. Less specific specialized natural products include flavonoid glycosides from various aglyca (Benayad et al., 2014).

Fenugreek is increasingly being consumed in the form of young, germinated seeds (sprouts) as an excellent source of nutrients, including proteins (Shakuntala et al., 2011). In fact, fenugreek is one of the major legume (Fabaceae) sprouts (Farag et al., 2021a). A study on 13 germinating seeds showed that total phenolic content on a dry weight (DW) basis increased during sprouting in darkness in all tested legumes, including fenugreek (Cevallos-Casals and Cisneros-Zevallos, 2010). Other studies on fenugreek have come to similar conclusions regarding total phenolic constituent and antioxidant activity (Pandey & Awasthi, 2015) and total flavonoid content (Saleh et al., 2019). However, there is conflicting evidence (Al-Juhaimi et al., 2016), and there are limited studies that provide further insight. A HPLC quantification study reported the appearance of minor phenolic constituents, including gallic acid and tyrosol, and an increase in caffeic acid, syringic acid and the flavonoid rutin during fenugreek germination (Oufquir et al., 2020). Another study showed that darkness and light conditions change the accumulation kinetics of the flavonoids vitexin and isovitexin in fenugreek sprouts, but no comparison was carried out with the dormant seed (Kalinová et al., 2021).

1.2. Chemical characterization methods of legume seeds and sprouts

Recently, the pitfalls associated with the use of total phenolic assays and total flavonoid assays have prompted questioning of their usefulness (Granato et al., 2018). It has been proposed to move towards LC-MS methods for profiling and quantification of phenolics and antioxidants. Widely used spectrophotometric determinations referred to as "total phenolic assays" actually measure thiols, a number of vitamins and inorganic ions as well (Everette et al., 2010).

Detailed data and high-coverage profiling methods for fenugreek are lacking, however. In addition to the lack of data on sprouts, studies on *Trigonella* seeds have focused primarily on single or only a few compounds, such as vitexin and isovitexin (Kalinová et al., 2021), caffeic acid, syringic acid and rutin (Oufquir et al., 2020), 4-OH-isoleucine (Farag et al., 2017; Gowtham et al., 2022; Singh et al., 2020; Wadhwa et al., 2022) or trigonelline (Wadhwa et al., 2022; Kowmudi et al., 2020). On the other hand, broad-coverage studies on sprouts (Benayad et al., 2014; Farag et al., 2021a) and seeds (Król-Kogus et al., 2020; Singh et al., 2020) are limited to screening without any validation or quality control assessments. This is in part because of the poor availability of authentic standards for steroid saponins and flavonoids. In addition, several screening studies have only provided data on specialized metabolites (Benayad, Gómez-Cordovés, & Es-Safi, 2014; Król-Kogus et al., 2020).

More detailed information has been published to date on metabolite changes during sprouting in other legumes. From the HPLC-UV or LC-MS studies on germinating legumes including *Vicia faba* (Mekky et al., 2020), *Vicia umbellata* (Li et al., 2018), various soybean cultivars (Guzmán-Ortiz et al., 2017; Krishnappa et al., 2017; F. Wang et al.,

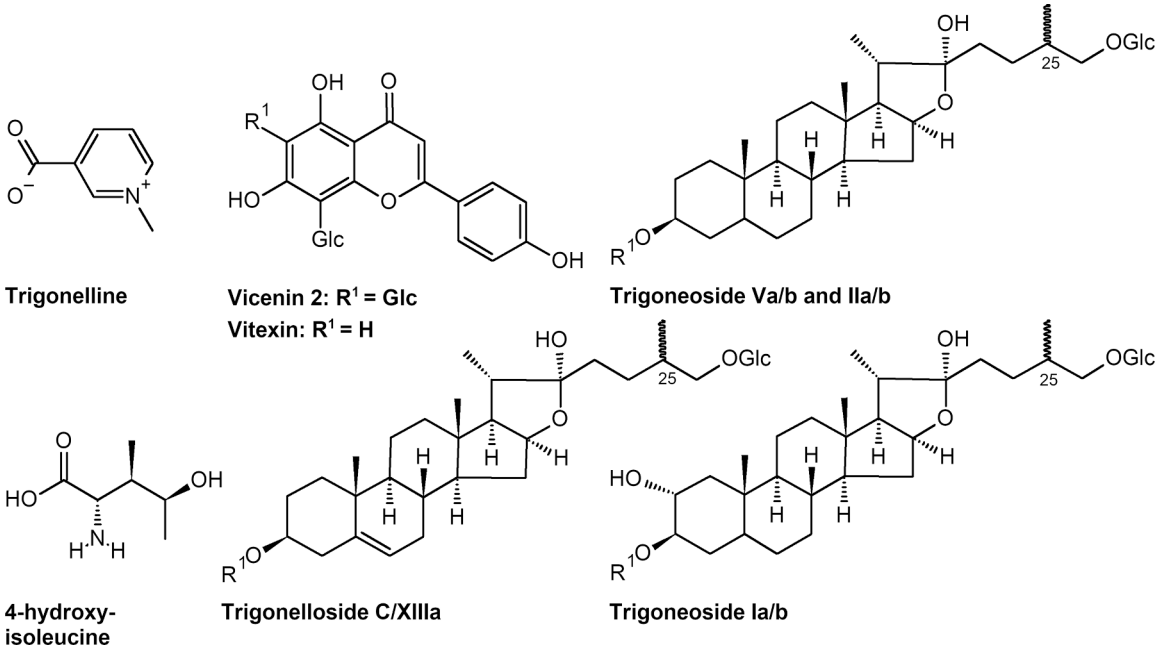

Fig. 1. Chemical structures of selected bioactive compounds of *Trigonella foenum-graecum*. Terpenoid (steroidal) saponins differ in glycosidic side chains and configuration of carbon 25, as follows: Trigoneoside Va/b: R^1 = Glc(1 → 6)[Xyl(1 → 4)]Glc(1 → 3)Glc(1 → 4)[Rha(1 → 2)]Glc, trigoneoside V/a: 25***S***, trigoneoside V/b: 25***R***; Trigoneoside IIa/b: R^1 = Xyl(1 → 6)Glc, trigoneoside II/a: 25***S***, trigoneoside II/b: 25***R***. Trigonelloside C: R^1 = Rha(1 → 4)[Rha(1 → 2)]Glc, 25***S***; Trigoneoside XIIIa: R1 = Glc(1 → 4)Glc(1 → 4)[Rha(1 → 2)]Glc, 25***S***; Trigoneoside Ia/b: R^1 = Xyl(1 → 6)Glc; trigoneoside I/a: 25***S***, trigoneoside I/b: 25***R***.

2015), and chickpeas (Xu et al., 2018), a common conclusion can be drawn which supports the issues raised by Granato et al. (2018). Namely, although an increase in total phenolic constituents is typically (but not always) detected during germination in darkness, there are many interesting trends at the level of individual metabolites that remain hidden and likely influence bioactivity of the products. A recent review of quantitative chromatographic measurements of various sprouts also shows a very mixed picture of the specialized metabolite kinetics during germination (Xu et al., 2020). Altogether, an in-depth characterization method for fenugreek seeds and sprouts is warranted.

1.3. The metabolomics approach: Advantages and caveats

Plant metabolomics is emerging as a viable alternative that combines most of the advantages of screening and quantitative determinations. The technique has become a mature approach in phytochemical analysis (Gonda, 2020), with a large number of publications every year. Its attractiveness stems from its high throughput: hundreds to thousands of *m/z*-retention time pairs (features) can be obtained from a single sequence, in contrast to quantitative techniques which rely on the use of authentic standards and are therefore typically limited to a few compounds. The most widely used technique for plant metabolomics is LC-ESI-MS/MS. This relatively rapid technique can detect a wide range of compounds in their native form and can identify hundreds to thousands of features, compared to only a few dozen with NMR metabolomics (Farag et al., 2021b).

However, special care must be taken while designing measurement sequences to avoid artefacts. While there are widely accepted protocols for the validation of methods with authentic standards, quality control in metabolomics is still a rapidly developing field (Martins et al., 2018). The lack of authentic standards requires workarounds to correct for drifts in instrument sensitivity and to identify peaks that originate from the sample (Dunn et al., 2011). A list of possible approaches and techniques that can address these issues was recently reviewed (Dudzik et al., 2018; Evans et al., 2020). In addition, the lack of instrument-to-instrument reproducibility of MS/MS spectra results in challenges when annotating compounds. Even though many compounds remain unannotated (Gonda, 2020), quality-controlled untargeted metabolomics studies can offer unprecedented insight into the composition of plant and food matrices.

1.4. Elicitation techniques

The increased biosynthesis of specialized natural products is usually referred to as elicitation. In sprouts, various strategies can be used for elicitation, including the use of stress hormones and their analogues, such as methyl jasmonate (MeJA) and salicylic acid (SA), as recently reviewed by Toro et al. (2021).

Data on the elicitation potential of fenugreek have been tested in only a few cases. These studies focused on a single compound or compound class. For example, in a callus culture, both MeJA and SA caused enhanced accumulation of trigonelline (Beygi et al., 2021), while MeJA also successfully increased diosgenin production in fenugreek sprouts (Chaudhary et al., 2015; De and De, 2011). Additional data on whole plants is also available (Irankhah et al., 2020).

Again, in-depth data are lacking for fenugreek; more information is available on other legumes. Successful enhancement of the biosynthesis of total polyphenols and flavonoids as well as individual specialized metabolites has been reported in rice bean (*Vigna umbellata* L.) sprouts growing in darkness and induced by 0.1–1 mM MeJA (Li et al., 2018). In another LC-MS study (Li et al., 2017), germinating mung beans were treated with 0.001–10 mM MeJA. Although 72-hour samples showed a 15 % reduction in total phenolic compounds, individual compounds showed 0.59- to 3.43-fold changes, clearly showing the advantages of high specificity methods.

1.5. Aims

Our objectives in the present work were: (1) chemical characterization of germinating fenugreek (*Trigonella foenum-graecum* L.) seeds (sprouts) using a food metabolomics approach, and (2) an attempt to increase the amount of specialized metabolites in fenugreek sprouts by methyl jasmonate and methyl salicylate. As a prerequisite, the performance of the applied LC-ESI-MS/MS method was also assessed, using a widely applied metric to assess method performance for 4-hydroxyisoleucine and trigonelline, as well as up-to-date quality control metrics for untargeted metabolomics data.

2. Materials and methods

2.1. Chemicals

All reagents used were of at least analytical quality. 2*S*,3*R*-4-hydroxyisoleucine and trigonelline of analytical standard quality were from Sigma Aldrich (St. Louis, MO, USA). Hydrogen peroxide, methanol and methyl jasmonate (MeJA) were purchased from the same company. Pharmaceutical-grade methyl salicylate (MeSA) was purchased from a local pharmacy. Type I water (18.2 MΩ), purified by a Zeneer Power I water purification system (Human corporation, Seoul, Korea) was used throughout the study. HPLC-MS grade acetonitrile, water and formic acid were purchased from Fisher Scientific (Geel, Belgium).

2.2. Fenugreek sprouts and hormone treatments

Pharmaceutical-grade fenugreek seeds (KräuterMix, Germany; with a certificate of analysis) were used for the germination study. Typical germination rate was proven to be 99–100 %. About 100 seeds were surface sterilized in 5 % H_2O_2 for 5 min, thoroughly washed in sterile type II water and subsequently allowed to germinate on a sterile filter paper in a sterile Petri dish for 72 h at 26 ± 0.5 °C in darkness. The sterile filter paper contained 1, 10 or 100 mM MeJA or MeSA dispersed in water; controls contained water. For each planned sampling time (24, 48 and 72 h) and treatment combination, 4 biological replicates were obtained.

2.3. Sample extraction

As germinated seeds turned out to be very difficult to homogenize in a frozen state, swollen or germinated seeds were frozen in liquid nitrogen, lyophilized, pre-crushed in mortar with the aid of liquid nitrogen, and finally homogenized with Retsch MM 300, using a 12 mm stainless steel ball in a 10 mL stainless steel grinding jar, at 28 Hz frequency, for 45 s, at room temperature. An accurately weighed 25 mg of dry powder was extracted with 1000 μL methanol in a tightly sealed plastic test tube in a block heater for 20 min at 70 °C. Methanol was chosen for because it has high polar compound coverage (Creydt et al., 2018), but it is not expected to dissolve galactomannans at all. Extracted samples were mixed, centrifuged, 25-fold diluted with methanol and filtered on 0.22 μm pore PTFE membranes before measurement.

2.4. Instrumentation and LC-MS measurement

For LC-MS measurements, an UHPLC system (Dionex Ultimate 3000RS) coupled to a Thermo Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, USA) with an electrospray ionization source (ESI) was used. Measurement parameters were the same as in our recent untargeted metabolomics work on horseradish roots (Plaszkó et al., 2022a), with modifications detailed below. The Orbitrap was operated in full MS mode at *m/z* range 125–1875 and FWHM resolution 35,000, with polarity switching enabled during all quantitative measurements. From each sample, we injected 1 μL.

2.5. Method performance assesment (4-hydroxyisoleucine and trigonelline)

To assess method performance, seven-point calibration curves of 4-hydroxyisoleucine and trigonelline were constructed in the range 1–10 μg mL^{-1}. Ty-pically-two calibration curves were measured within a measurement day, at the beginning of the sequence and at the end of the sequence.

The raw abundance data from the same concentration points were used to calculate precision (intraday standard deviation). Data from neighboring measurement days were used to calculate interday precision. Accuracy was calculated by spiking a 25-fold diluted pooled QC sample (made of real samples) with 2.5 or 5 μg mL^{-1} (approximately + 75–100 %, +150–200 %) authentic standards and calculating the recovery. This was done on different measurement days, with at least 2 replicates per level on each day. LOD was estimated from baseline noise level of ions of interest, using the linearity equation.

2.6. Targeted metabolomics

A manual search for bioactive compounds was conducted in mzMine 2.53 (Pluskal et al., 2010) by checking for compounds described in (Farag, Rasheed, Kropf, & Heiss, 2016; Kang et al., 2013; Król-Kogus et al., 2020; J. Wang et al., 2017), followed by a targeted peak detection of the files using the same software with an intensity tolerance of 50 %, noise level 10, *m/z* tolerance of 5 ppm and retention time tolerance of 0.2 min. Peaks were joined with the join aligner, at 5 ppm *m/z* tolerance and 0.15 min retention time tolerance, with equal weight for both parameters. Compounds previously described for fenugreek included mainly flavonoid glycosides (Farag et al., 2016) as well as a diverse set of furostanol saponins trigoneosides and trigofoenosides (Farag, Rasheed, Kropf, & Heiss, 2016; Kang et al., 2013; Król-Kogus et al., 2020; J. Wang et al., 2017). Area under curve values for authentic standards were also calculated using this approach.

2.7. Untargeted metabolomics

XCMSOnline 2.7.2 (XCMS 1.47.3) was used for peak detection in untargeted metabolomics (Gowda et al., 2014). The parameters of peak detection have already been described (Plaszkó et al., 2022a).

2.7.1. Quality control samples, their usage and measurement sequence design

Metabolomics QC samples were prepared according to the most typical protocol, the pooled QC approach ("intra-study QC samples") (Dudzik et al., 2018; Evans et al., 2020) by mixing equal volumes of samples, using a single sample replicate from each treatment group. This meant a sample from all combinations of the agent, concentrations and time points. A 25-fold dilution was used, unless otherwise indicated. Run order randomization and measurement sequence design (blanks, QC blocks) was accomplished using established works in the field (Dudzik et al., 2018; Evans et al., 2020) and has been described in detail recently (Plaszkó et al., 2022a).

2.7.2. Feature filtering and adjustments to changes in instrument sensitivity

Features were filtered for precision and linearity before feeding to downstream analyses (Broadhurst et al., 2018; Dunn et al., 2011). Only features with $R^2 > 0.8$ or 0.9 were kept for further analysis of biological phenomena. This step also discards features that are present in significant amounts in the blank sample. For all compounds, a LOESS (low-order nonlinear locally estimated smoothing) approach was used to correct for minor deviations in sensitivity (Dunn et al., 2011). Essentially, this expresses all abundances as fold-changes where the reference (1.00) is the feature abundance in a pooled QC sample. The amount of extracted dry weight was also corrected for in this step. Additional details can be found in our recent paper (Plaszkó et al., 2022a).

2.8. Putative identification of QC-passed features

2.8.1. MS/MS

Targeted fragmentation of all features of interest was accomplished with similar parameters, except that the mass range was reduced to include only the ions of the inclusion list and that positive and negative ion mode data were recorded separately.

A list of candidate features was prepared from (1) features that were expected from literature data and found in QC samples, (2) features that passed metabolomics QC (section 2.7.2.). The list of candidates was split into inclusion lists so that at most 5 co-eluting features were included in a single list, resulting in good coverage of all list members around their peak tops. Depending on the inclusion list overlaps, the top 2–5 features were selected for fragmentation at 30 normalized collision energy (NCE) in a rotation scheme (loop count = 5, topN = 5). Maximum ion collection time was set at 250 ms.

2.8.2. Putative compound class annotation of unknown compounds

MS^2 spectra were harvested from raw measurement files with the R package CluMSID (Depke, Franke, & Brönstrup, 2019). For each feature, the top 10 MS^2 spectra were used to generate a consensus spectrum, which was exported from R and subsequently imported into SIRIUS 4.9.9 (Dührkop et al., 2019) for annotation. The CANOPUS algorithm of SIRIUS was thereafter used to generate Classyfire hierarchical classes of organic compounds (Djoumbou Feunang et al., 2016; Dührkop et al., 2021) for each feature separately.

2.9. Statistical analysis

Resulting tables from XCMS were further processed using custom scripts in R 4.1.2., using the packages including ggplot2 3.3.4 (Wickham, 2016), foreach 1.5.1 (Daniel et al., 2022). Statistical inference for testing the difference between treatments and sampling points was conducted using the Scheirer-Ray-Hare test (a two-way extension of the Kruskal Wallis test) (Mangiafico, 2022). Wilcoxon tests were then used as post-hoc tests to compare (1) control zero time samples with other control samples and (2) control samples to samples of various treatments, pooled from all time points. To assess significant differences, the Benjamini Hochberg adjusted *p*-values were used at a false discovery rate alpha = 0.05 (Storey & Tibshirani, 2003). The procedure was applied separately for the untargeted metabolomics dataset, the targeted metabolomics dataset and the post-hoc tests.

3. Results and discussion

3.1. Identified Trigonella foenum-graecum compounds

As summarized in Table 1, a total of 31 compounds could be identified based on MS/MS literature data (Farag, Rasheed, Kropf, & Heiss, 2016; Kang et al., 2013; Król-Kogus et al., 2020; J. Wang et al., 2017). Flavonoid derivatives and steroidal saponins were characterized in negative ion mode, with a few exceptions (Table 1). Altogether 7 flavonoid glycosides (typically C-glycosides of apigenin) were detected. The highly diverse steroid saponins were represented by 19 members. The typical number of saccharide units was 3–5; diosgenin and yuccagenin were the most frequently encountered aglyca.

From the MS/MS data, chemical classes (according to the Classyfire hierarchy (Djoumbou Feunang et al., 2016)) were computed with Canopus (Dührkop et al., 2021). Of 237 candidates from untargeted metabolomics, 75 features could not be assigned to any meaningful class, and 29 were assigned to very high-level natural product-like groups, such as "glycosides" or "phenolic substances" (Table S3). This is likely due to poor S/N ratio of the MS/MS spectra.

On the other hand, 17 putative phenolic specialized metabolites, including 7 flavonoid glycosides, a stilbene glycoside and a chalcone glycoside, were successfully annotated. Manual comparison with

Table 1
Compounds of *Trigonella foenum-graecum* described in the literature, detected in tested samples with the proposed method. Error is *m/z* difference between found and expected value. References: (1) (Farag et al., 2016), (2) (Kang et al., 2013), (3) (Król-Kogus et al., 2020), (4) (Wang et al., 2017). Rt, retention time; Ref, reference. Compounds identified as MSI level 1 (with authentic standards) are marked with an asterisk.

Name	Rt (min)	*m/z* $[M-H]^-$	*m/z* $[M+H]^+$	Formula [M]	error (ppm)	Fragments	Ref.
Trigonelline *	3.03	–	138.06	$C_7H_7NO_2$	−2.92		*
4-hydroxyisoleucine *	3.11	–	148.1	$C_6H_{13}NO_3$	−1.81	130.0864, 113.0599, 102.0918, 84.0812, 74.0242	*
N-gamma-glutamyltyrosine	10.78	–	311.12	$C_{14}H_{18}N_2O_6$	−1.64	294.0968, 182.0812, 165.0545, 136.0757	(1)
Hydroxybenzoic acid pentosyl-hexoside	11.02	431.12	–	$C_{18}H_{24}O_{12}$	0.81	137.0231, 93.033	(1)
Vicenin 2	12.02	593.15	–	$C_{27}H_{30}O_{15}$	0.94	503.1201, 473.1088, 413.0879, 383.0774, 353.0667	(1)
Chrysin-6,8-C-dihexoside	12.44	577.16		$C_{27}H_{30}O_{14}$	0.12	503.1207, 473.1079, 457.1143, 383.077, 353.0666	(1)
Apigenin-C-xyloside-C-xyloside	12.5	533.13	–	$C_{25}H_{26}O_{13}$	−0.4	473.1092, 443.0986, 383.0774, 353.0667	(1)
Apigenin-8-C-rhamnoside-6-C-hexoside	12.62	577.16	–	$C_{27}H_{30}O_{14}$	0.29	487.1249, 457.1145, 383.0774, 353.0669	(1)
Vitexin	12.8	431.1	–	$C_{21}H_{20}O_{10}$	−0.51	353.0682, 341.0666, 323.0562, 311.0562, 283.0613, 269.0456	(1)
Apigenin-6,8-C-dipentoside methyl ether	12.86	547.14	–	$C_{26}H_{28}O_{13}$	−0.67	487.1272, 457.1148, 383.077, 353.0663	(1)
Neogitogenin-dirhamnosyl-glucoside	12.86	–	1065.55	$C_{51}H_{84}O_{23}$	−1.19	903.4948, 759.3806, 595.3867, 433.3309, 289.2166, 271.2053, 253.1947	(2)
Diosmetin-8-C-glucoside	12.87	461.11	–	$C_{22}H_{22}O_{11}$	0.25	371.0776, 341.0665, 298.0479	(1)
Diosgenin-diglucosyl-rhamnoside	12.91	–	1047.54	$C_{51}H_{82}O_{22}$	−1.53	944.6538, 885.487, 577.3756, 415.3211, 271.2057, 253.1947	(2)
Trigoneoside Ia/b	12.93	905.47	–	$C_{44}H_{74}O_{19}$	−5.75	773.4384, 611.3799, 449.3267	(3), (4)
Diosgenin dirhamnosyl hexoside	12.93	–	889.48	$C_{45}H_{76}O_{17}$	−1.23	583.3028, 451.2682, 415.3184, 289.2159, 271.2054, 253.1949	(1)
20(22)-ene-1,26-diol-Proto-yamogenin-rhamnosyl-dihexoside	13	–	901.48	$C_{45}H_{72}O_{18}$	−1.1	597.331, 447.3929, 287.2008, 253.1951	(1)
Trigoneoside XIIa/b	13	–	903.49	$C_{45}H_{74}O_{18}$	−2.26	597.3265, 451.266, 433.331, 415.3192, 289.216	(1)
Trigoneoside Va/b	13.06	1519.68	–	$C_{68}H_{112}O_{37}$	−2.18	1387.6372, 1063.5331, 901.4797, 755.4221, 593.3682	(3), (4)
Trigoneoside VIIIb	13.07	1521.69	–	$C_{68}H_{114}O_{37}$		1389.6482, 1065.5443, 903.4928, 757.4378, 323.0975	(4)
Diosgenin 3-O-glucoside	13.07	–	577.37	$C_{33}H_{52}O_8$	−0.77	415.3201, 271.2054, 253.1948	(1)
Trigoneoside XIIIa/XIIIb	13.18	1225.58	–	$C_{57}H_{94}O_{28}$	−2.97	1179.5808, 1063.5337, 901.4821, 755.4243, 593.364	(3), (4)
Proto-neogitogenin-triglucosyl-rhamnoside	13.27	1065.54	–	$C_{51}H_{86}O_{23}$	−5.24	903.4942, 757.4372, 595.3845, 461.3199	(4)
Yuccagenin-dirhamnosyl-glucoside	13.27	–	1047.54	$C_{51}H_{82}O_{22}$	−1.72	944.6538, 739.4293, 577.3733, 431.1351, 287.2006, 269.1906	(2)
Trigonelloside C	13.31	1047.53	1049.55	$C_{51}H_{84}O_{22}$	−6.97	901.4824, 755.424 (negatve ion mode) 1013.5281, 887.4991, 743.3854, 433.3307, 289.2163 (positive ion mode)	(3), (4)
Trigoneoside IIa/b	13.34	889.47	–	$C_{44}H_{74}O_{18}$	−7.07	757.4396, 595.3877, 433.3303	(3), (4)
Proto-yamogenin-diglucoside	13.35	935.49	–	$C_{45}H_{76}O_{20}$	−0.07	889.4809, 757.4382, 727.4282, 595.3855, 433.3314	(2)
25(27)-ene-Proto-neogitogenin-diglucosyl-rhamnoside	13.38	1079.54	–	$C_{51}H_{84}O_{24}$	8.31	1033.5199, 901.4808, 755.4203	(2)
Trigofenoside A	13.44	901.47	–	$C_{45}H_{74}O_{18}$	−6.2	755.4242, 689.4534, 593.3663, 533.5217	(3), (4)
Furost-5-ene-tetrol-O-trihexoside methyl ether	13.45	947.49	–	$C_{46}H_{78}O_{20}$	0.14	901.4816, 755.423, 593.3703, 431.315	(1)
Trigoneoside IIIb	13.48	903.49	–	$C_{45}H_{76}O_{18}$	−7.79	757.4357, 595.3901	(3), (4)
Dihydroxy-octadecadienoic acid	16.63	311.22	–	$C_{18}H_{32}O_4$	2.14	293.2127, 275.2018, 253.1807, 235.1696, 223.1697, 87.0436	(1)

literature data in this group has reinforced the putative identifications of the chalcone and previously identified flavonoid C-glycosides in several cases.

Additionally, 28 putative terpenoids, including 19 glycosides of steroid aglyca, could be annotated. Manual verification was even more reassuring this time: in 13 cases, fragmentation either found natural products that have already been described (5 cases) or compounds that can be described as derivatives of these products (8 cases). The latter group includes a putative diosgenin-derivative (605.2993@13.05) and two putative trigoneoside Ia/b derivatives (919.4836@12.98 and 981.4922@12.82). The aglyca could typically be identified; therefore, the sugar side chain is most likely modified compared to the previously described compounds.

Primary metabolite classes were also assigned to compounds in a total of 88 cases. Putative annotations included 40 saccharides, 25 amino acid-related compounds, 11 peptides, 8 lipid derivatives, 4 nucleotides and two hormones (Table S3).

3.2. Method performance

3.2.1. Trigonelline and 4-hydroxyisoleucine

Starting the elution gradient with 100 % water + 0.1 % HCOOH resulted in excellent separation of 4-hydroxyisoleucine and trigonelline from non-retained compounds, without major changes in elution characteristics of other compounds. Good to excellent recoveries were achieved for trigonelline and 4-hydroxyisoleucine-spiked samples,

respectively (Table S1). This shows the lack of matrix-borne ion suppression phenomena in the retention time region.

Simultaneous quantification of native non-derivatized 4-hydroxyisoleucin and trigonelline is not straightforward. Previous studies have either admitted to being uncapable of accomplishing this (Farag et al., 2016) or provided incomplete method performance metrics. Examples of this include a screening study that could detect 4-hydroxyisoleucin and flavonoids (Keskes et al., 2018) and an MRM-based LC-MS method (Singh et al., 2020). In the latter, both 4-hydroxyisoleucine and trigonelline co-eluted with other non-retained compounds and the solvent front, likely resulting in very strong ion suppression effects and, subsequently, inaccurate quantitation (Furey et al., 2013). On the other hand, excellent validated LC-MS methods for determination of 1–3 compounds have also been published (Gowtham et al., 2022; Wadhwa et al., 2022; Kowmudi et al., 2020). However, these were not tested for assessment of other *Trigonella* compounds.

3.2.2. Metabolomics approach

A total of 31 features (Table 1) were integrated with mzMine in a targeted peak search; 28 of these could pass the QC filtering (RSD for precision < 20 % and R^2 for linearity > 0.9, Table S2). Robustness was also reflected in the median R^2 of 0.978 and a median RSD value of 8.36 % for these features (Table S2).

Automatic peak finding with XCMS resulted in 3521 features, of which 1842 remained after filtering out isotopes and adducts. QC filtering (RSD < 30 %, $R^2 > 0.8$), yielded a total of 237 features that passed quality control (Table S3).

Overall, we believe that the current approach is well suitable for the characterization of fenugreek seeds and sprouts because (1) it is capable of detecting a wide range of compounds including 4-hydroxyisoleucine and trigonelline (2) its quantification abilities are supported by traditional or metabolomics-tailored performance metrics.

3.3. Changes in feature abundances during germination

Based on targeted metabolomics, a non-significant increase on a DW basis was observed for most compounds: the median fold change compared to dormant seeds was 1.01, 1.15 and 1.36 for 24, 48 and 72 h samples, respectively. This could be due in part to the degradation of storage carbohydrates as previously described in other studies (Bakhshy et al., 2019; Pandey & Awasthi, 2015).

The results from the untargeted metabolomics dataset provided a much deeper insight into the changes: they showed that there is a variety of trends for individual compounds during germination in darkness, in the absence of externally added hormones (Figs. 3-4). A set of 64 features (of 237) showed significant changes at different sampling time points ($p_{adj} < 0.05$, Table S3), including 12 amino acid derivatives, 2 lipids, a nucleotide, 4 peptides and 9 saccharides, 2 steroid saponins, but no flavonoids.

3.3.1. Changes in primary metabolism

Targeted analysis showed that dihydroxy-octadecadienoic acid varied significantly across different sampling time points ($p_{adj} = 0.0039$, Fig. 2d, Table S2). This suggests that the plant uses some lipoxigenase during germination to access its lipid storage pool (Feussner et al., 1995).

Untargeted metabolomics showed that increases and decreases were detectable within most groups of primary metabolites (Fig. 4. a-e). For example, 72 h versus 0 h abundance changes in amino acid derivatives, peptides and saccharides fell in the 0.09- to 22.25-fold, 0.93- to 478.79-fold and 0.36- to 941.58-fold ranges, even though all of these groups were characterized by an overall increase: median fold-change values were 1.645, 1.76 and 1.425, respectively (Fig. 4. a, d, e; Table S3).

Germination resulted in overall massive changes in the plant metabolome (Fig. 4h). Most of the affected features were primary metabolites (Table S3, Fig. 4. a-e), which is consistent with an LC-MS study on *Vigna umbellata* (Li et al., 2019).

Some compounds showed no or almost no presence in seeds, followed by a rapid onset of biosynthesis within the first 24 h (Fig. 4). This group included a carboxylic acid derivative (170.0813@11.2, Fig. 3b) and a saccharolipid (433.1349@11.81, Fig. 3c). In germinating soybeans, various primary metabolites, including a few amino acids, fumarate, ascorbate, galactitol, free fructose and glycerol, have presented similar kinetics (Gu et al., 2017).

Other compounds were present in significant amounts in dormant seeds, but a subsequent rapid degradation occurred during the first 24 h. Examples include a putative phosphocholin-type lipid (520.3406@17.5) and a putative amino acid derivative (478.2934@17.16, Fig. 3d). Similar behavior has been observed for some primary metabolites in soybeans (Gu et al., 2017), for saccharides, amino acids, choline metabolites and tricarboxylic acid metabolites in *Vigna radiata* (L.) R. Wilczek (X. Wu et al., 2020) and for sugars, citric acid and a few other organic acids in *Vicia faba* L. (Mekky et al., 2020).

Another group, including a few *N*-acyl amines or amino acids, showed a transient increase in controls, as exemplified by 410.3266@19.62 (Fig. 3h) and 408.3117@19.61. A similar pattern has been observed for galactinol and galactitol in soybeans during the first 4 days of sprouting (Gu et al., 2017).

3.3.2. Changes in specialized metabolites

The targeted analysis showed that the changes in the key bioactive compounds 4-hydroxyisoleucine and trigonelline were not significant ($p_{adj} > 0.05$, Fig. 2a, c, Table S4). Their amount increased by 35.5 % and 33.3 % by 72 h, which can in part be explained by mass effects (Bakhshy et al., 2019; Pandey & Awasthi, 2015). The compounds were present at 0.37 % and 0.29 % DW in the seeds, in line with literature data (Avalos-Soriano, De la Cruz-Cordero, Rosado, & Garcia-Gasca, 2016; Laila et al., 2019).

Untargeted metabolomics data showed that most specialized metabolites increase in abundance during germination (Fig. 4. f-g): nine flavonoids and their subgroup flavonoid 8-C glycosides showed fold-change medians of 1.49 and 2.05 by 72 h compared to the dormant seeds. Vitexin, a major C-glycoside, registered a 2.62-fold change (Table S2). Although these values are lower than the changes reported for total phenolic constituent changes (Cevallos-Casals and Cisneros-Zevallos, 2010), the major trends are in line with previous reports. Detailed LC-MS analysis is important (Granato et al., 2018), since large changes in individual compound concentrations may remain hidden behind a relatively stable total polyphenolic or flavonoid content. This has been shown for anthocyanins in germinating seeds of *Phaseolus vulgaris* L. (López et al., 2013), polyphenolics in germinating seeds of *Vigna radiata* (Krishnappa et al., 2017) and total isoflavones in soybeans (Chen & Chang, 2015; Huang et al., 2014). In the study by Chen & Chang (2015), an unchanged overall amount of isoflavones was the net result of 0.71- to 6.92-fold change values for individual compounds. Due to the effects of light demonstrated in germinating soybean varieties (Kim et al., 2006), only literature on legumes germinating in darkness is considered here for comparison.

The fold change range of 1.19–2.77 for individual flavonoids (Table S2) in germinating fenugreek is not high compared to that in other legumes: an LC-MS study (Z. Wu et al., 2012) revealed that by day 3, about 1400-fold increases in formononetin can be observed in germinating chickpeas (*Cicer arietinum* L.). Another LC-MS study on germinating *Vicia faba* (Mekky et al., 2020) reported a 17- to 36-fold increase in flavonoids, with a set of 6 putative flavone glycosides, 3 flavone aglyca, 5 putative flavonol glycosides (but not rutin) and 2 flavonol aglyca showing increased concentrations. In a study on various Chinese soybean varieties, total and individual isoflavone contents were quantified by LC-MS (F. Wang et al., 2015). The authors found a 1.24- to 1.86-fold increase in total isoflavones by day 7.

Conversion of flavonoid subclasses to others was expected based on the literature, as detailed below. This phenomenon should have been

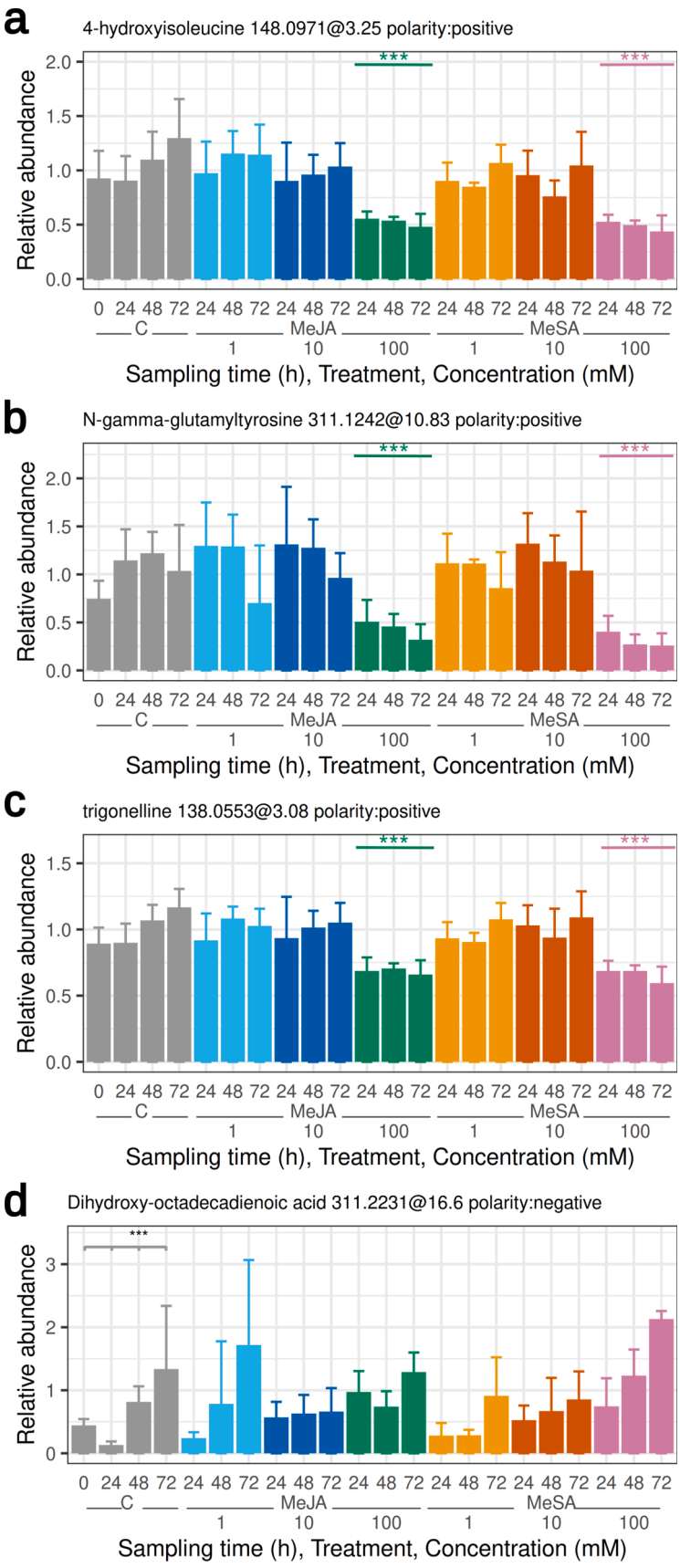

Fig. 2. Changes in abundances of selected compounds in germinating fenugreek seeds, germinating in darkness for 72 h, assessed by targeted metabolomics. The seeds were treated with 1, 10 or 100 mM concentrations of MeJA or MeSA. Header shows compound name, followed by *m/z* @ retention time. All presented compounds show significant differences after Benjamini-Hochberg correction, either across hormone treatments (a-c) or sampling time (d). Black asterisks denote significant differences across sampling time points; colored asterisks denote treatments that are significantly different from the control (***, $p_{adj} < 0.001$; **, $p_{adj} < 0.01$; *, $p_{adj} < 0.05$). For statistical tests, see 2.9.

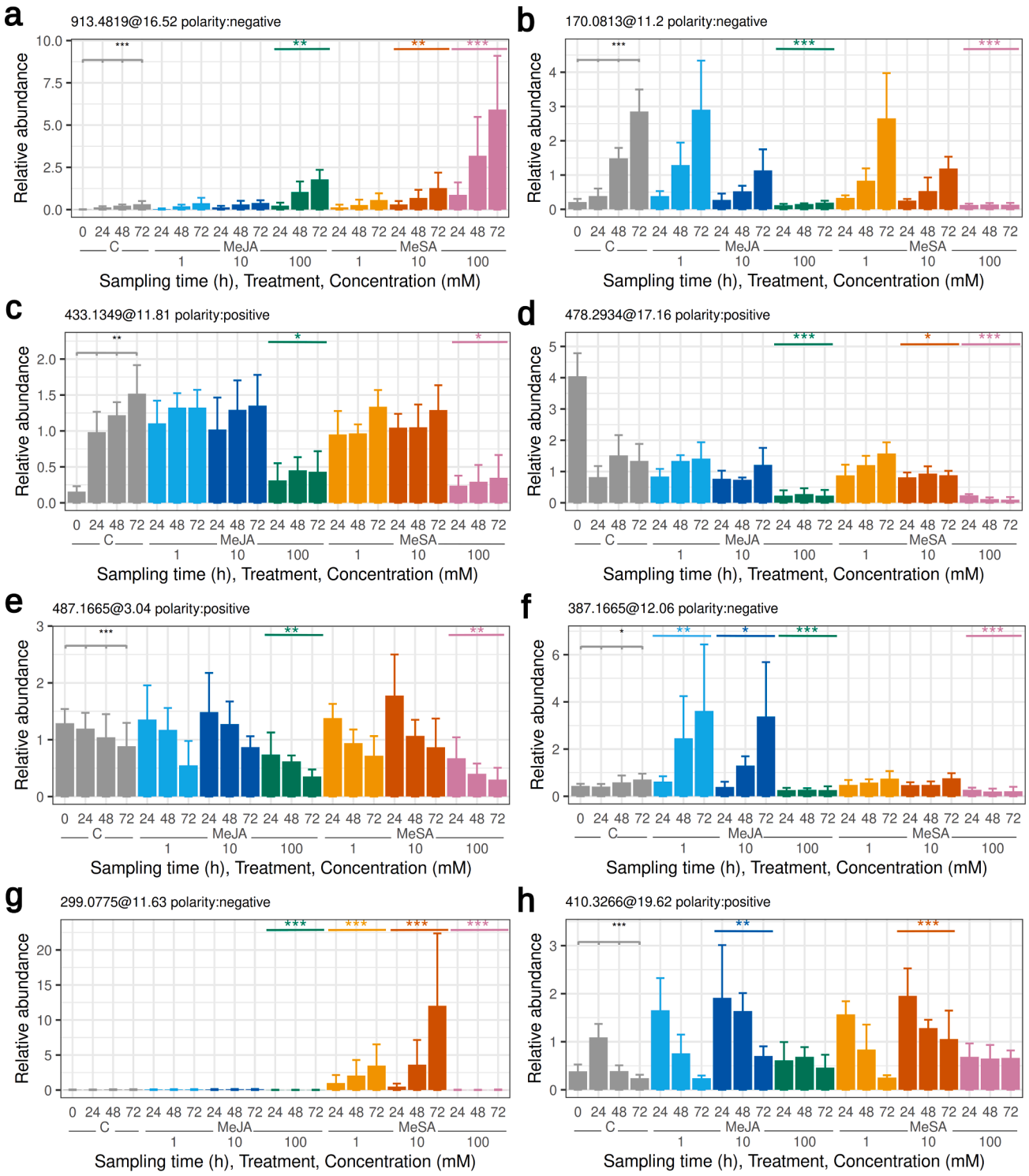

Fig. 3. Changes in abundances of selected features in germinating fenugreek seeds, germinating in darkness for 72 h, assessed by untargeted metabolomics. The seeds were treated with 1, 10 or 100 mM concentrations of MeJA or MeSA. Headers show *m/z* @ retention time. All presented features show significant differences after Benjamini-Hochberg correction across hormone treatments; compounds in subplots a-c and e-h also exhibit differences for sampling time. Black asterisks denote significant differences between sampling time points; colored asterisks denote treatments significantly different from the control (***, $p_{adj} < 0.001$; **, $p_{adj} < 0.01$; *, $p_{adj} < 0.05$). For statistical tests, see 2.9.

present as various trends for individual flavonoids, but we did not detect any sign of this (Fig. 4f). A study by F. Wang et al. (2015) found an increase in malonylglucosides of isoflavonoids as well as a reduction in the concentrations of beta-glucosides and aglyca in various germinating soybean cultivars. These results were confirmed in a study by Guzmán-Ortiz et al. (2017), which showed an overall increase in isoflavones (aglyca daidzein and genistein) and mixed effects on phenolic compounds during a 6-day germination of soybeans. Chickpea seeds have also shown considerable variability regarding the changes in phenolic compound composition. This was demonstrated in an LC-ESI-MS study by Xu et al. (2018) which detected increases in 7,3′,4′-trihydroxyflavone and hesperitin but a decrease in isoflavones (glycitein). A detailed study

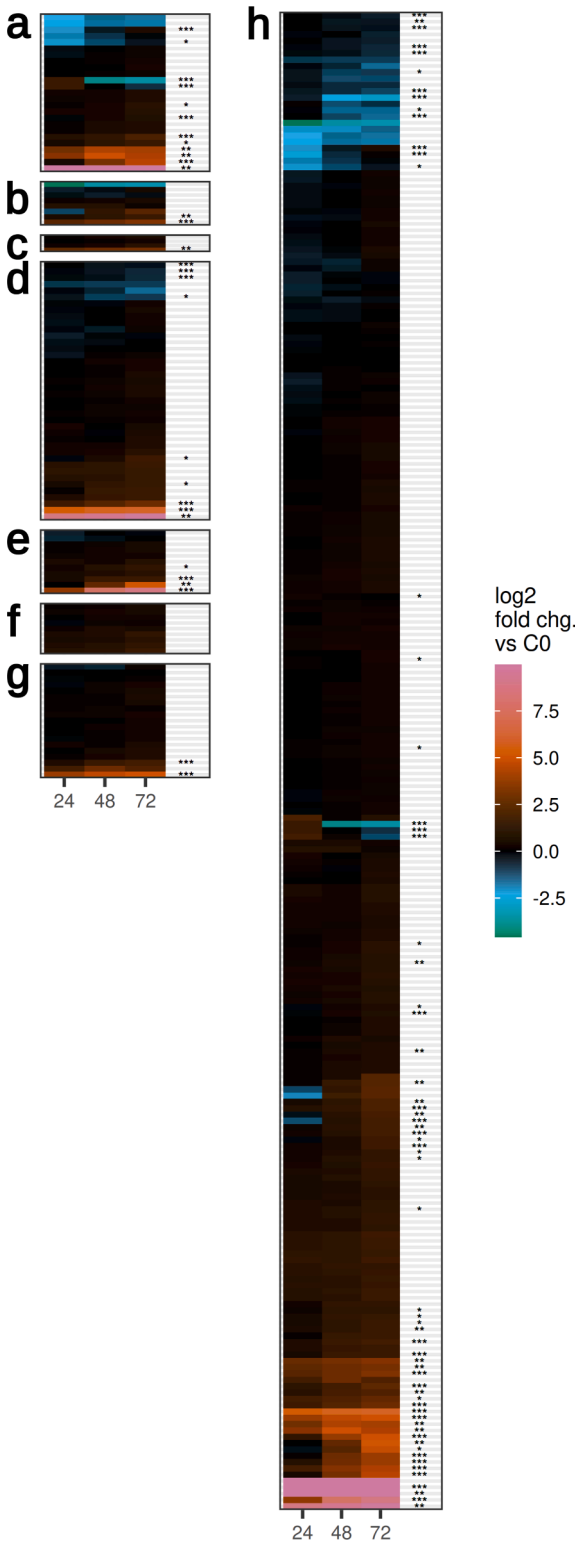

Fig. 4. Changes in abundance of compounds in germinating fenugreek seeds compared to dormant seeds, assessed by untargeted metabolomics. The sprouts were germinated under hormone-free conditions in darkness and sampled at 24, 48 and 72 h (x axes). Compounds were grouped according to compound type and sorted with hierarchical clustering. Subplots: a., amino acid derivatives; b., lipids; c., nucleotides; d., peptides; e., saccharides; f., flavonoids; g., steroid saponins; h., whole metabolome. Significant differences between sampling time points are shown as ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. For *p*-values, consult Table S3. For statistical tests, see 2.9. (A color version is available online.)

on *Vigna umbellata* (Li et al., 2018) also found a combination of increasing and unclear trends for flavonoids and isoflavonoids during germination.

Steroid saponins showed a fold change range of 1.08–31.86 with a median of 1.44. Moreover, two of the features (727.4275@16.21 and 913.4819@16.52 (Fig. 3a)) showed a significant increase ($p_{adj} < 0.001$, Table S3). The amount of literature on saponin changes during germination is much more limited than that on flavonoids but usually presents increases. A total saponin assay for soybeans germinated in darkness for 7 days found a 35 % increase in total saponins (Chen & Chang, 2015). Soyasapogenol B, but not A, was found to be increased at 60 h in three cultivars of germinating soybeans (Rupasinghe et al., 2003). In an LC-MS study on *Vicia faba* (Mekky et al., 2020), a 2- to 12-fold change in saponins during germination was observed, and increases were reported for 7 saponins. Conflicting evidence also exists: no changes in saponins were observed during germination of *Cicer arietinum* and *Lens culinaris Medik.* (Ruiz et al., 1996).

3.4. Effects of methyl jasmonate and methyl salicylate treatments on the metabolome of fenugreek sprouts

The rationale behind testing MeJA and MeSA was the attempt to further increase the levels of specialized metabolites during germination. Successful attempts have been already described in germinating legumes (Li et al., 2017) and other plants (Park et al., 2013).

The targeted metabolomics approach demonstrated that 17 of 28 features showed significant differences across various treatments (Scheirer-Ray-Hare test, Table S2, Fig. 2). However, a closer look shows that, in contrast to expectations, all of these phenomena represent a reduction of abundance compared to control conditions, especially in 100 mM MeJA and 100 mM MeSA treatments at 72 h (Table S2, Fig. 2). Median fold changes in the targeted metabolomics dataset were 0.825, 0.83, 0.75, 0.86, 0.96 and 0.69 for 1, 10 and 100 mM MeJA and 1, 10 and 100 mM MeSA treatments, respectively.

The reduction in also apparent in the untargeted metabolomics dataset: 67.1–83.1 % and 64.1–83.5 % of compounds showed a reduction compared to the controls in 100 mM MeJA and MeSA treatments at different sampling time points (Fig S1, Table S3–S4), resulting in median fold change values of 0.68 and 0.61 for 100 mM MeJA and MeSA, respectively. While it is reasonable to assume that general stress would interfere with the biochemical machinery and result in a slower usage of storage nutrients (Bakhshy et al., 2019), various trends (Fig S1) and the heterogeneity within various compound class responses (Fig. 5) need further discussion.

3.4.1. Effects of stress hormones on primary metabolites in germinating fenugreek seeds

Hormone treatments, especially at 100 mM concentrations, seemed to interfere with primary metabolism according to targeted metabolomics results: the concentration of *N*-gamma-glutamyltyrosine was significantly reduced ($p_{adj} < 0.001$, Fig. 2b). This is also apparent on a larger scale in the untargeted metabolomics data (Fig. 5 a-e, Fig. S1), providing more detailed insight. A set of 149 features (of 247) showed significant differences in various treatments ($p_{adj} < 0.01$, Table S3). These differences were mainly due to the decrease in the 100 mM MeSA and 100 mM MeJA treatments, which were significantly different from controls for of 85 and 97 features, respectively.

The inhibitory effect of high hormone concentrations was exemplified by a carboxylic acid derivative (170.0813@11.2, Fig. 3b), a saccharolipid (433.1349@11.81, Fig. 3c) and a putative amino acid derivative (478.2934@17.16, Fig. 3d). What is more, a clear dose–response relationship could be found in a few instances, for example in the case of a monoacylglycerol (369.2641@15.75, $p_{adj} = 2.93E-05$), a linoleic acid derivative (323.2582@20.75, $p_{adj} = 5.89E-10$), and an alpha amino acid derivative (412.3064@14.54, $p_{adj} = 3.69E-04$). On the other hand, the levels of several amino acid derivatives and a few lipids,

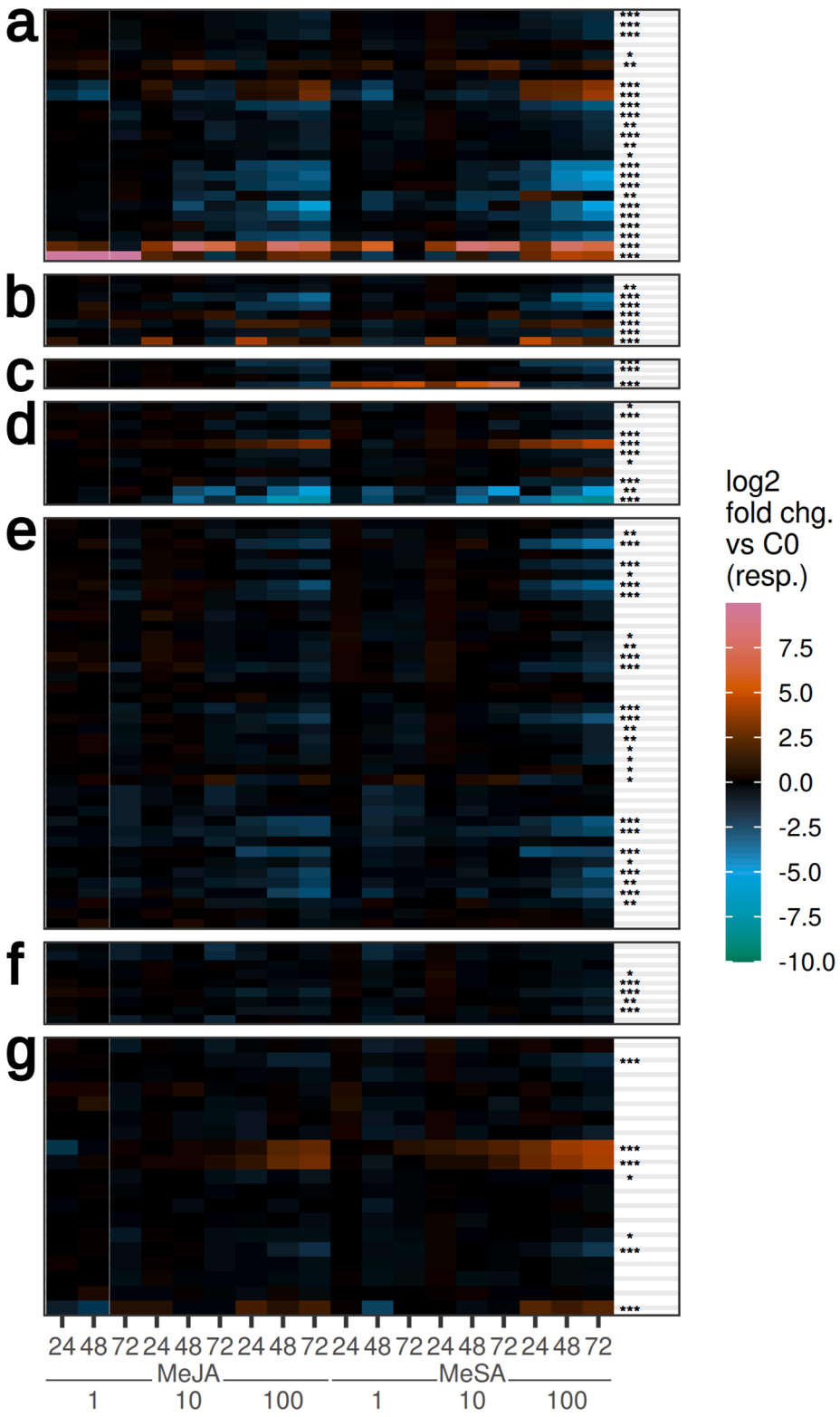

Fig. 5. Changes in abundance of compounds in hormone-treated fenugreek seeds compared to control sprouts, assessed by untargeted metabolomics. Treatment was either methyl jasmonate (MeJA) or methyl salicylate (MeSA) at different concentrations (1, 10, 100 mM). Sampling time was 24, 48 and 72 h (x axes). As a reference, controls of the same sampling time were used. Compounds were grouped according to compound type and sorted with hierarchical clustering. Subplots: a., amino acid derivatives; b., lipids; c., nucleotides; d., peptides; e., saccharides; f., flavonoids; g., steroid saponins. Significant differences between treatments are shown as ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. The heatmap for the whole metabolome is shown in Fig. S1. For *p*-values, consult Table S3. For statistical tests, see 2.9. (A color version is available online.)

nucleotides and saccharides increased significantly (Fig. 5a-c, e).

Altogether, 92 % of putative amino acid derivatives, 87 % of lipids, 82 % of peptides and 64 % of saccharides showed a significant response ($p_{adj} < 0.05$). Almost all saccharides and peptides and the majority of amino acid derivatives responded with decreased abundance to 100 mM treatments, while lower dose treatments ranged from no change to medium reductions. This is in line with the findings of Li et al. (2019), who treated *Vigna umbellata* sprouts with 0.1 mM MeJA. The authors found 29 significantly different features which included mostly primary metabolites, namely amino acids, amines, sugars and sugar alcohols, organic acids and fatty acids.

3.4.2. *Effects of stress hormones on specialized metabolites in germinating fenugreek seeds*

As shown by targeted metabolomics, the overall reduction in compound abundances included a mixture of various responses. The increase

in 4-hydroxyisoleucin and trigonelline seen during germination was clearly inhibited by 100 mM MeJA and MeSA: compared to respective controls, only 0.34- to 0.42-fold and 0.45- to 0.55-fold abundances were measured ($p_{adj} < 0.001$, Table S2, S4, Fig. 2a, c), respectively. On the other hand, other compounds, including some steroid saponins, showed elicitation phenomena. For these, up to 1.4- and 1.62-fold change values were observed for 100 mM MeJA and MeSA versus controls, respectively (Table S2). To obtain a more detailed picture, we once again move on to data obtained from untargeted metabolomics. These reinforced the findings of targeted metabolomics (Fig. S1). Altogether, 7 of 19 steroid saponins and 5 of 9 flavonoids showed significant effects from the hormone treatments (Fig. 5f-g).

Some steroid saponins (424.3055@16.89, 727.4275@16.21 and 913.4819@16.52 (Fig. 3a)) showed spectacular elicitation phenomena, with fold changes in the 100 mM MeSA treatment being 4.6, 17.01 and 18.65 compared to control (Fig. 5.g, $p_{adj} < 0.001$). On the other hand, other terpenoid saponins showed severe reductions, with values as low as 0.29 compared to controls (981.4922@12.82).

In contrast to expectations, flavonoids completely failed to be elicited in these treatments: median fold change versus controls was 0.75 and 0.68 after treatment with 100 mM MeJA and MeSA. No individual flavonoid showed increased biosynthesis. Interestingly, in contrast to what is seen for many primary metabolites, lower doses did not perform better either (Table S3). In the literature, mixed effects are found in studies using quantification procedures. Li et al. (2019) showed that 0.1 mM MeJA lowers the increase in abundance of daidzein amd genistein versus control in 48 h germinated rice bean seeds, even though there is an increase in sprouts compared to the dormant seeds. This phenomenon corresponds exactly with our findings on fenugreek. In another study from the same authors (Li et al., 2018), *Vigna umbellata* sprouts grown in darkness were treated with 0.1–1 mM MeJA. This study found a decreased concentration for isoquercitrin and glycitein and an increased concentration of rutin and kaempferol-3-O-rutinoside by day 6. Other flavonoids showed trends that were erratic and not as clear. On the other hand, another study (Gómez et al., 2022) successfully used a range of 0.04–4.4 mM of MeJA to increase biosynthesis of various phenolics including isoflavones and derivatives in the Fabaceae plants *Phaseolus vulgaris*, *Glycine max* L. and *Vigna radiata*. The differences could be due to differences in genotype and experimental variables.

Although methyl jasmonate and methyl salicylate often play distinct roles in plant defense (Plaszkó et al., 2022b), only four compounds showed specific elicitation by one of the hormones. MeJA-specific induction was shown for the putative aromatic compounds (possibly C_6C_1 glycosides) 373.1869@13.16 and 387.1665@12.06 (Fig. 3f). Examples for MeSA-specific induction included the purine nucleotide metabolic intermediers 299.0775@11.63 (Fig. 3g) and 315.0726@10.83.

As the 100 mM treatments typically flattened trends regardless of direction (compare Fig. 3 d, e and h), a possible explanation is the interference with general metabolism, normal germination and development. Overdosing plant stress hormones often leads to decreased functioning or even necrosis of plant tissues in tissue cultures (Gueven & Knorr, 2011). Under the current experimental conditions, contrary to expectations, no general elicitation occurred. As the increased biosynthesis of specialized metabolites is limited to a few examples, the qualitative pattern of specialized metabolites was left more or less intact.

4. Conclusions

Fenugreek is a functional food of broad scientific interest, and it is increasingly being consumed in the form of sprouts. Despite this popularity, there is a lack of comparative chemical data on fenugreek sprouts. We have presented and used an LC-ESI-MS method capable of simultaneous determination of 4-hydroxyisoleucin and trigonellin and of assessment of other important bioactive natural products from chemically distinct bioactive compound classes.

Germination of fenugreek in darkness resulted in changes in both primary and specialized metabolites. The amount of key bioactives (trigonelline, 4-hydroxyisoleucine) increased by 35.5 % and 33.3 %, respectively. that the amount of steroid saponins, however, showed considerable variation: while the median fold change was 1.49, some compounds increased in abundance over 30-fold. The increase in the abundance of flavonoids fell in the 1.19–2.77-fold-change range, with a median of 1.44.

Treatments with widely applied stress hormones methyl jasmonate and methyl salicylate, on the other hand, failed to further increase the specialized metabolites. With the exception of three steroid saponins, the amounts decreased. At the same time, these treatments interfered with normal development and caused significant effects on primary metabolism.

Altogether, as the specialized metabolite pattern is the same, the bioactivity linked to them is likely to be relatively intact. Consequently, fenugreek sprouts can be considered for incorporation into products if small molecule bioactives are desired, but the natural fibers are undesirable because of their interference with technological procedures. Since the increases in primary metabolites, including amino acid derivatives and saccharides, were much higher, additional experiments are warranted on the changes in the nutritive potential of fenugreek and perhaps other legume sprouts.

The quality controlled untargeted metabolomics approach proved to yield excellent insight into the metabolic changes of fenugreek germination. We believe that similar approaches should be used to revisit formerly examined food matrices to get a higher resolution picture on differences in the pattern of bioactive metabolites and nutritive and antinutritive constituents.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112347.

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