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Original article

Exploring the anti-α-amylase activity of flavonoid aglycones in fabaceae plant extracts: a combined MALDI-TOF-MS and LC–MS/MS approach

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Summary A combination of TLC-bioautography, MALDI-TOF-MS and LC–MS/MS methods was used to identify flavonoids with anti-α-amylase activity in extracts of *Lathyrus pratensis* L. (herb), *L. polyphillus* L. (fruits), *Thermopsis lanceolata* R. Br. (herb) and *S. japonica* L. (buds). After the TLC-autobiography assay, substances with anti-amylase activity were identified by MALDI-TOF-MS followed by confirmation of the result by LC–MS/MS. Results of the study revealed that the flavonoids apigenin, luteolin, formononetin, genistein and kaempferol display marked anti-α-amylase activity. Formononetin showed the largest activity. Compared with LC–MS/MS, MALDI-TOF-MS is a quick and convenient method; results can be obtained within minutes; and only minor sample amounts are required which allows us to analyse mixtures of substances without preliminary separation. However, the inability to distinguish between isomers is the main limitation of the method.

Introduction

Diabetes mellitus is a group of disorders associated with elevated blood glucose levels (Alam *et al.*, 2014). According to the WHO, the number of people affected was 422 million in 2014. Type 2 diabetes is a disease whose occurrence can be prevented or delayed through diet, physical activity and regular checkups (Sarwar *et al.*, 2010). The increase in type 2 diabetes is due to the availability of sweet, high-starch foods, which cause a spike in blood glucose after consumption. Inhibiting the activity of digestive enzymes that degrade carbohydrates, such as α-amylase, is one way to prevent diabetes.

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Experiments showed that flavonoids can inhibit α-amylase (Sawicka *et al.*, 2021, Proença *et al.*, 2020) and can help to lower postprandial blood glucose levels (Proença *et al.*, 2019). Fabaceae plants are known to have a very high flavonoid content (Budantsev, 2010). In our study, we have chosen four species, with two used both in traditional medicine and as components of food supplements (*Sophora japonica L.* and *Thermopsis lanceolata* R. Br.), as well as further two that are not included in the pharmacopoeia (*Lathyrus pratensis* L. and *Lupinus polyphyllus* L.) yet. Possible anti-diabetic properties of these plants or related species were investigated.

The flower buds and other parts of *S. japonica* L. are used in both Russian and traditional Asian medicine. Due to their high rutin content, the flower buds

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are used to treat hemorrhagic disease. Flavonoids are one of the major class of compounds present in the plant and 39 flavonoids and 41 isoflavonoids were identified (He *et al*., 2016). The potential anti-diabetic properties of Sophora extract were already reported. Sophora flavonoids can significantly improve basal glucose uptake in HepG2 cells *in vitro* (Chen *et al*., 2010) and lower glucose levels in rats with streptozotocin-induced diabetes (Miao *et al*., 2014). The flavonoids from Sophora were also shown to inhibit the digestive enzyme α-amylase, but only one flavonoid, namely kaempferol glycoside with pronounced activity, was identified (Zhang *et al*., 2020).

Thermopsis lanceolata R. Br. is used in medical practice because of its alkaloid thermopsin, which has an expectorant effect. Also, *T. lanceolata* R. Br. has a high content of cytisine which possesses a selective binding affinity for neuronal nicotinic acetylcholine receptors and it is therefore used to treat tobacco dependence (Karnieg and Wang, 2018). As far as we know, the flavonoid content of *T. lanceolata* R. was not studied. The flavonoid composition of the related plant *Thermopsis alterniflora* R. Br. was investigated. This plant is also used as a source of cytisine (Grudzinskaya *et al*., 2020). Flavonoid aglycons were found in the aerial part of the plant: luteolin, chrysoeriol, formonetin, apigenin and cynaroside (Kotenko *et al*., 2001).

Lathyrus pratensis L. was considered a source of biologically active compounds (Solovyeva *et al*., 2019), including flavonoids. According to studies, the aerial part of the plant contains quercetin, luteolin, formononetin, apigenin (Zaichikova *et al*., 2001), kaempferol and their derivatives (Llorent-Martínez *et al*., 2016).

Lathyrus pratensis L. crude extracts showed an antidiabetic effect through inhibiting enzymes α-amylase and glucosidase (Llorent-Martínez *et al*., 2016). However, the compounds responsible for this effect have not been identified.

Lathyrus polyphyllus L. belongs to the large genus *Lupinus*, originating in North and South America (Valtonen *et al*., 2006). Plants of this genus contain several isoflavonoids, genistein and its isoprenylated form are prevalent in fruits and pods (Katagiri *et al*., 2000). Hypoglycemic activity of substances of Australian lupin is reported (Mazumder *et al*., 2021).

Due to their high content of protein, fats and phenolic compounds, lupine seeds can be considered a functional food (Farag *et al*., 2019), but they contain quinolizidine alkaloids (Mancinotti *et al*., 2022) which impart a bitter taste and are toxic to humans. Therefore, a cleaning step is necessary to remove these alkaloids from the seeds before they can be consumed (Yeheyis *et al*., 2011).

Thin layer chromatography (TLC) coupled with bioautography assay makes it possible to separate a crude extract from the plant material and to highlight components with biological activity (*e.g*. ability to inhibit α-amylase) (Legerská *et al*., 2022). Mass spectrometry, particularly MALDI-TOF-MS, is used for the analysis of high-molecular compounds, such as biomolecules, proteins and more complex chemical compounds. Several studies have proven that MALDI-TOF-MS is a satisfactorily working method for analysing flavonoids (Ye *et al*., 2013).

MALDI-TOF-MS offers several advantages, in contrast to LC–MS/MS (Xin *et al*., 2011), it does not require sophisticated sample preparation methods; it is a fast and convenient method, the results can be obtained within minutes and only very low sample amounts are required. Moreover, the MALDI method allows us to analyse mixtures of substances without their preliminary separation (Fougère *et al*., 2019). However, an inability to discriminate between related chemical structures can occur due to the mass (m/z) similarity of the flavonoids themselves.

In this study, we have established the possibility of using TLC coupling with the MALDI-TOF-MS method and LC–MS/MS as reference tool to determine some flavonoid aglycones of *L. pratensis* L. (herb), *L. polyphillus* L. (fruits), *T. lanceolata* R. Br. (herb) and *S. japonica* L. (buds), respectively, that can inhibit the α-amylase activity.

Materials and methods

Reagents and materials

Genistein (98.8%), apigenin (98.2%), luteolin (98.4%), kaempferol (100.0%) and formononetin (98.6%) were supplied from Cayman Chemical (Ann Arbor, Michigan, USA). Acarbose was purchased from Sigma–Aldrich (Milano, Italy).

Chromatographic separation was carried out using Merck silica gel 60 F254 plates, 7 × 10 cm; silica gel 60 (Darmstadt, Germany). Hexane, ethyl acetate, acetic acid and the auxiliary substances iodine, potassium iodide, potassium dihydrophosphate and potassium hydrophosphate for 6.86 buffer solution preparation (analytically pure grade) came from Chimmed, Russia. Dinitrosalicylic acid (DNS-reagent) was purchased from PanReac (Barcelona, Spain). α-amylase was purchased from Sigma–Aldrich (Milano, Italy).

The MALDI matrices 2,5-dihydroxyacetophenone (DHAP, 699832) and α-Cyano-4-hydroxycinnamic acid, ultrapure (CHCA, 70990), were purchased from Sigma–Aldrich (Darmstadt, Germany). Methanol (1428) and acetonitrile (2697) (both LC–MS grade) were purchased from Th. Geyer GmbH & Co. KG (Renningen, Germany). TFA (LC–MS grade, 85 183) was purchased from Sigma–Aldrich (Darmstadt, Germany). Ammonium citrate dibasic (09833) was

purchased from Sigma–Aldrich (Darmstadt, Germany). Ultrapure reagent water for buffers and solutions was obtained from a Milli-Q Synthesis A10 water purification system (Merck Millipore, Darmstadt, Germany); KGaA (Darmstadt, Germany). Ethyl acetate, n-hexan, acetic acid, iodine and potassium iodide were purchased from Sigma–Aldrich (Darmstadt, Germany). Formic acid (FA; MS grade) was from Sigma–Aldrich (Darmstadt, Germany). Acetonitrile (Ultra gradient HPLC grade) and methanol (Ultra gradient HPLC grade) were purchased from J.T.Baker (Deventer, Netherlands).

Samples and preparation of the extracts

In a local pharmacy, a dry sample of *S. japonica* (buds) was purchased. Fresh herbs of *T. lanceolata* R. Br. and *L. pratensis* L. (herb), and fruits and pods of *L. polyphyllus* L. were collected from the Botanical Garden of Sechenov First Moscow State Medical University, Moscow, Russia in July 2021. The raw materials were dried in a dark place at room temperature and cut to a particle size of 3–5 mm. The dried and cut plant materials were extracted with 95% ethanol by maceration for 3 days; the ratio of raw material to ethanol was 1:1.5. The obtained extracts were stored at 4 °C prior to further analysis.

Bioautography assay

The study was conducted according to the methodology described by Agatonovic-Kustrin *et al*., (2017) with minor modifications (Chapter S1–S3).

Determination of α-amylase inhibition activity of crude extracts and flavonoid aglycons

For quantitative determination of anti-α-amylase activity, the enzyme was diluted in water at a ratio of 1:2000 (*v*/*v*) for crude extracts and of 1:50 000 (*v*/*v*) for flavonoid aglycones (for more information see the Chapter S9). All solutions were incubated at 37 °C before use. The anti-α-amylase activity of crude extracts and flavonoids, the method described by *Daoudi et al*. (Daoudi *et al*., 2020) with slight modifications was used. Briefly, 100 µL of an individual flavonoid solution with varying concentrations (1.5, 1.0, 0.5 and 0.1 mg/mL) was mixed with 50 µL of enzyme solution, and 160 µL of phosphate buffer solution (Sigma–Aldrich (Darmstadt, Germany) at pH 6.86 was added. The mixture was shacked and incubated at 37 °C for 20 min. After adding 400 mL of 1% starch solution, the mixture was shacked and incubated for 15 min. Thereafter, 600 µL of the resulting solution was added to 300 µL of DNS solution in a glass tube and boiled in a water bath for 10 min and 5.9 mL of water was added to the solution and stirred. Ultimately, the absorbance of the reaction mixture was measured at 540 nm.

α-amylase inhibitory capacity (%) was calculated by the formula:

$$\alpha\text{−amylase inhibition}\% = \left[1-\left(\frac{A_{\text{sample}}-A_{\text{blank}}}{A_{\text{test}}-A_{\text{control}}}\right)\right]\cdot 100\%,$$

where A_{sample} is the absorbance of the solution containing the sample, enzyme solution, starch solution and DNS-reactive; A_{blank} is the absorbance of the solution containing the sample, starch solution and DNS-reactive, without enzyme; A_{test} is the absorbance of the solution containing solvent (instead of the sample), starch solution, enzyme and DNS; A_{control} is the absorbance of the solution containing solvent (instead of the sample), water (instead of enzyme), starch solution and DNS-reactive. IC_{50} values were calculated based on % α-amylase inhibition vs. individual flavonoid concentrations. Acarbose was used as a reference inhibitor (Additional information see in Chapter S9, Fig. S10).

TLC-MALDI-TOF-MS

MALDI-TOF the mass spectra were acquired using a Bruker Reflex III MALDI mass spectrometer from Bruker-Daltonik (Bremen, Germany) operated with a nitrogen laser and at 20 kV acceleration voltage.

The TLC plate was pre-washed with the mobile phase hexane: ethyl acetate: glacial acetic acid (20:9:1) and dried. On the starting line, 50 µL of the extract was applied in stripes of 1 cm, with a distance of 0.5 cm between the stripes. The extracts were developed in ascending order. The chromatography was stopped when the mobile phase passed 80% of the plate height, and the plate was removed from the chamber and air-dried.

For MALDI-MS measurements, the sample spots were scratched from the TLC plate and mixed with 200 µL MeOH. The slurry was mixed for 10 min, centrifuged on a table centrifuge and afterwards, the supernatant was applied *via* dry droplet method with CHCA matrix on the target. Measurement was performed using a Bruker autoflex maX (Bruker Daltonik GmbH, Bremen, Germany) with a MALDI laser source (355 nm, 2000 Hz) and a TOF detector. The MS data were obtained in positive reflection mode with an average of 500 laser shots on the spot. The mass to be examined was set as parents' mass with a precursor mass window of $m/z \pm 2$. Argon was used as the collision gas.

For the measurement on the TLC plate, the TLC plate was attached to a plain microscope slide (J. Melvin Freed; 75 × 25 mm)[2] by an electrically conductive

Table 1 α-amylase inhibitory effects of medicinal herbs extracts.

	***L. pratensis* L.**	***S. japonica* L.**	***T. lanceolata* R. Br.**	***L. polyphillus* L.**
α-amylase concentration	1:2000	1:2000	1:2000	1:20 000
Inhibition, %	29.1 ± 9.5%	28.6 ± 8.5%	25.1 ± 4.6%	98.0 ± 9.8%

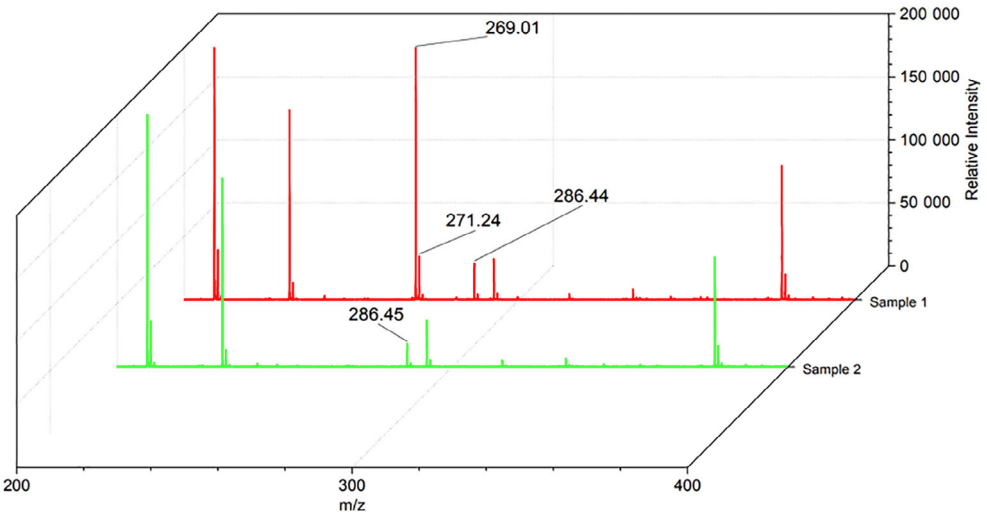

Figure 1 Positive ion MALDI-TOF mass spectra of flavonoid sum from *L. polyphyllus* L. representation of m/z value of spot zones (sample 1 with $R_f = 0.2$, sample 2 with $R_f = 0.59$).

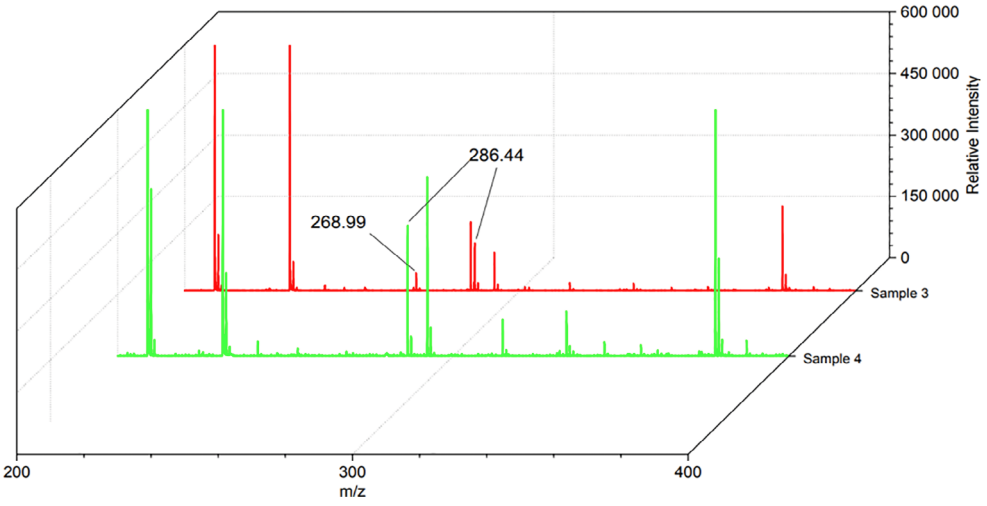

Figure 2 Positive ion MALDI-TOF mass spectra of flavonoid sum from *L. pratensis* L. Representation of m/z value of spot zones (sample 3 with $R_f = 0.2$, sample 4 with $R_f = 0.59$).

double-sided adhesive tape 9713 (3 M XYZ-axis, nickel-plated carbon scrim). Next, the matrix, consisting of 4 mL of 25 mg 2,5-dihydroacetophenone in ethanol and 1 mL of 9 mg diammonium hydrogen citrate in MilliQ lab water, was applied by an airbrush (Airbrush Pistole Kit AT-AK-o1, Agora-Tec, Schmalkalden, Germany). Then, the airbrush was applied to an XYZ table (Probot Micro Fraction Collector, Thermo Scientific, USA). The Chip was placed 5 cm under the airbrush tip and 2 bar pressure was applied to the airbrush gun. The XYZ table moved 100 times the chip under the airbrush. The sample

spots were marked with a pencil and measured with the same method as previously described (see Table S2, Fig. S4–S7, Table S8).

LC–MS/MS

A Nexera LC-30 (Shimadzu, Japan) chromatographic system coupled with SPD-M20A diode array detector (Shimadzu, Japan) and LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Japan) was used for analysis. The chromatographic separation was performed on the Agilent Zorbax Eclipse XDB-C18 column (4.6*150 mm; 5 μm) at 40 °C. The conditions of the screening and confirmatory LC–MS/MS methods are presented in supplementary materials (Chapter S11, Table S12). Sample preparation for LC–MS–MS analysis is described in Chapter S13 and LC–MS–MS method validation Chapter S14, Fig. S15, Table S16.

Results and Discussion

Inhibitory activity of crude extracts and bioautography assay

Extracts of *L. pratensis* L., *S. japonica* L. and *T. lanceolata* R. Br. showed comparable anti-α-amylase activity, whereas *L. polyphillus* L. extract did

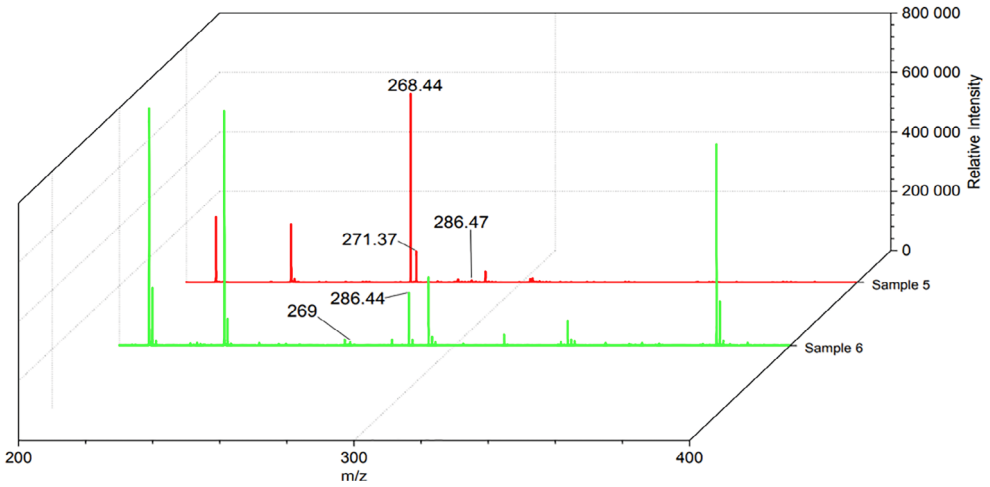

Figure 3 Positive ion MALDI-TOF mass spectra of flavonoid sum from *T. lanceolata* R. Br. Representation of m/z value of spot zones (sample 5 with $R_f = 0.36$, sample 6 with $R_f = 0.75$).

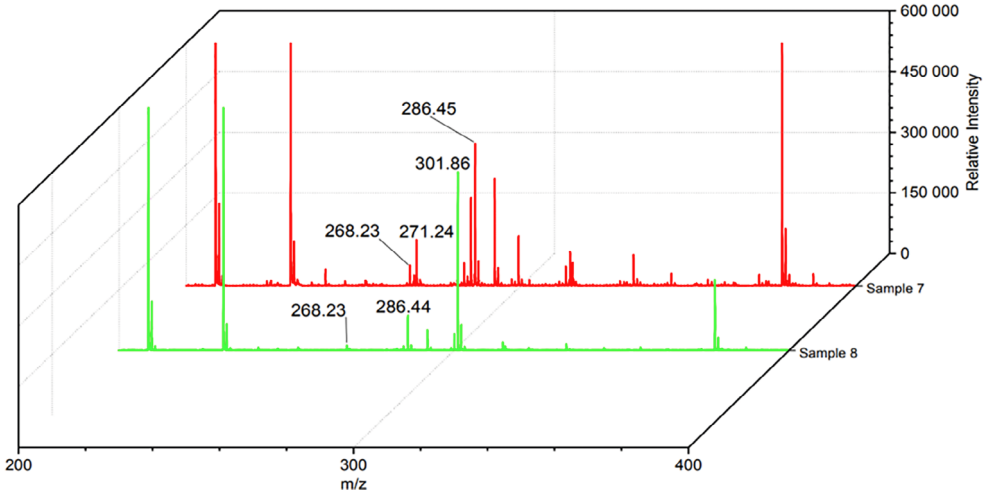

Figure 4 Positive ion MALDI-TOF mass spectra of flavonoid sum from *S. japonica* L. Representation of m/z value of spot zones (sample 7 with $R_f = 0.35$, sample 8 with $R_f = 0.19$).

not inhibit α-amylase activity at the selected concentration. Its anti-α-amylase activity was determined using an α-amylase solution diluted 10-fold (Table 1).

As a result of a bioautography assay, blue spots were observed on the chromatographic plate, which corresponded to the substances that have the ability to inhibit α-amylase. An example chromatogram is shown in Fig. S10.

MALDI-TOF-MS

Due to the porous surface of the TLC silica layer, a crystallisation of the matrix with the analytes is difficult to achieve. Nevertheless, some flavonoids could be identified by MALDI-TOF-MS (the details can be found in the Table S8). Therefore, MALDI-TOF-MS represents an easy method for the predevaluation and detection of flavonoids. The flavonoids genistein, apigenin, luteolin, kaempferol and formononetin could be identified. However, MALDI-TOF-MS, on the other hand, cannot distinguish between isomers with identical masses.

In the mass-spectrum of flavonoid sum from *L. polyphyllus* L., intense peaks were observed for ions with *m/z* 269.45 which corresponds to formononetin; *m/z* 271.24 which corresponds to genistein/apigenin, and *m/z* 286.44 which corresponds to luteolin/kaempferol (Fig. 1). The intense ion peak was observed in the mass spectrum of the studied sample of *L. pratensis* L. with the major peak at *m/z* 268.99 which corresponds to formononetin; *m/z* 286.44 which corresponds to luteolin/kaempferol (Fig. 2). In the mass-spectrum of flavonoid sum from *T. lanceolata* R. Br. L. intense peaks were observed for ions with *m/z* 286.47, which corresponds to luteolin/kaempferol and peaks *m/z* 268.44 which corresponds to formononetin and *m/z* 271.37 which corresponds to genistein/apigenin (Fig. 3). The intense ion peak was observed in the mass spectrum of the studied substance of *S. japonica* L. for ions with *m/z* 268.23 which corresponds to formononetin; *m/z* 271.24 which corresponded to genistein/apigenin; *m/z* 286.44 which corresponds to luteolin/kaempferol; *m/z* 301.86 which corresponded to quercetin (Fig. 4).

Table 2 Results of the preliminary identification of flavonoid aglycones' by LC–MS.

R_f of TLC spot	[M + H]	[M − H]	Supposed substance
L. polyphillus L.			
0.20	269.1	267.1	Formononetin
	271.1	269.1	Genistein/Apigenin
	287.1	285.1	Luteolin/Kaempferol
0.59	269.1	267.1	Formononetin
L. pratensis L.			
0.20	271.1	269.1	Genistein/Apigenin
	269.1	267.1	Formononetin
	287.1	285.1	Luteolin/Kaempferol
0.59	271.1	269.1	Genistein/Apigenin
T. lanceolata R.Br.			
0.36	271.1	269.1	Genistein/Apigenin
	269.1	267.1	Formononetin
	301.1	299.1	Chrysoeriol
0.75	269.1	267.1	Formononetin
	257.1	255.1	Liquiritigenin/Isoliquiritigenin
	271.1	269.1	Genistein/Apigenin
	287.1	285.1	Luteolin/Kaempferol
S. japonica L.			
0.19	269.1	267.1	Formononetin
0.35	257.1	255.1	Liquiritigenin/Isoliquiritigenin
	269.1	267.1	Formononetin

LC–MS/MS

The analysis of TLC spots samples with the screening LC–MS method demonstrated a multitude of *m/z* values that may be attributed to flavonoid aglycones (Table 2).

We observed a mixture of substances in each sample since the compounds with similar properties could not be completely separated on the TLC plate. We have carried out a preliminary identification of the substances in TLC spot samples using literature data (Fu and Wang, 2015) and the results of our previous studies (Adamov *et al.*, 2020, Struchkov *et al.*, 2019). We assumed the presence of apigenin, genistein, luteolin, kaempferol and formononetin in all the studied extracts. The screening LC–MS method, however, does not allow a fully reliable identification of these substances since the gradient elution program does not provide complete separation of flavonoid aglycones.

Table 3 Content of apigenin, genistein, luteolin, kaempferol and formononetin in plant material

	Concentration, μg/g				
Sample	Genistein	Apigenin	Luteolin	Kaempferol	Formononetin
L. polyphillus L.	64.0 ± 2.0	802 ± 41	50.5 ± 7.7	—	Trace
L. pratensis L.	—	16.2 ± 0.5	6.4 ± 0.1	—	Trace
T. lanceolata R. Br.	2412 ± 77	1286 ± 62	2140 ± 88	—	1297 ± 52
S. japonica L.	11.4 ± 1.0	6.2 ± 0.3	—	31.4 ± 7.8	1.3 ± 0.1

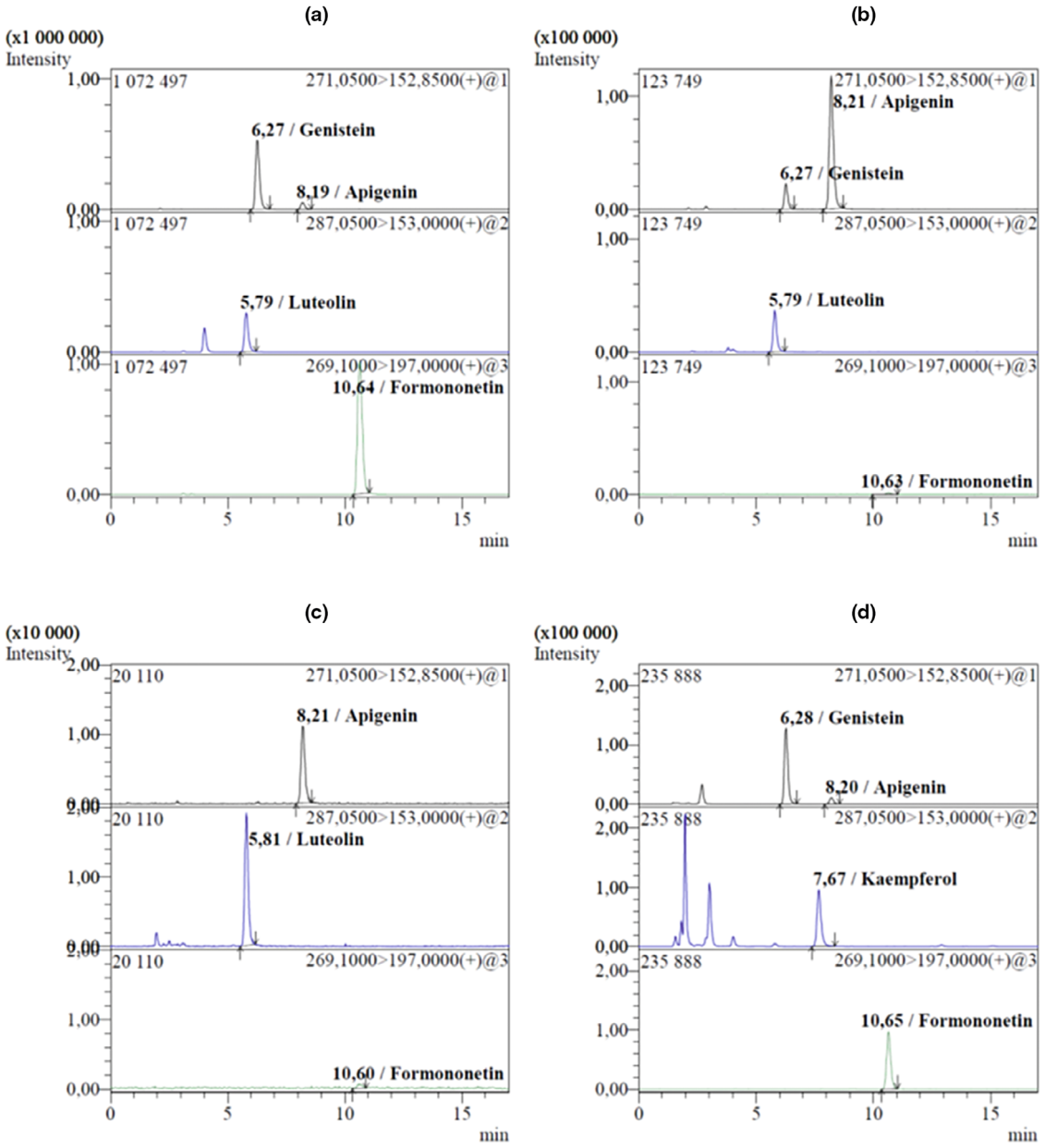

Figure 5 MRM chromatograms of A. *T. lanceolate* R. Br., B. *L. polyphillus* L., C. *L. pratensis* L., D. *S. japonica* L. extract samples.

For example, apigenin and genistein have identical retention times under the screening LC–MS method conditions. Moreover, the isomer pairs apigenin/genistein and luteolin/kaempferol have both the same molecular weight and the same fragmentation pathways when analysed in MRM mode. Thus, we replaced acetonitrile with methanol in the mobile phase and optimised the gradient elution program to achieve complete chromatographic separation. Ultimately, we used the modified method to confirm and quantify apigenin, genistein, luteolin, kaempferol and formononetin in plant extracts, and the corresponding results are shown in Table 3 and Fig. 5.

Inhibitory activity of identified flavonoids against α-amylase

All flavonoids were found to have the ability to inhibit α-amylase. The results are shown in Table 4.

Table 4 The IC_{50} values of individual flavonoids on inhibiting α-amylase activity compared to acarbose.

Component	IC_{50} (μg/mL)
Apigenin	37.13 ± 4.98
Luteolin	50.55 ± 8.42
Formononetin	48.30 ± 13.02
Genistein	53.67 ± 9.38
Kaempferol	30.83 ± 7.90
Acarbose	49.63 ± 7.24

The anti-α-amylase activity of flavonoid aglycones has been reported in numerous publications. For example, Sun *et al*. compare the ability of luteolin, kaempferol and other pomegranate rind compounds to inhibit salivary α-amylase (Sun *et al*., 2018). Zhang *et al*., (2017) cite the results of inhibition of porcine α-amylase by luteolin, apigenin, kaempferol and genistein compared to acarbose. Demir *et al*., (2019) describe the anti-α-amylase activity of formononetin and genistein, however, the authors do not specify the type of amylase used in their study. Since the authors of the papers mentioned here use different types of enzymes in their studies, the comparison results individually differ from article to article, however, the flavonoid aglycones in question display comparable anti-α-amylase activity to acarbose.

Conclusion

In the course of this study, we could prove that the use of the matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) method and LC–MS/MS allowed the preliminary characterisation of flavonoid mixtures in ethanol extracts of *L. praetensis* L. herb, *L. polyphillus* L. fruits, *T. lanceolata* R. Br. herb, *S. japonica* L. buds without first separating them into separate components with minimal sample preparation. The inability of MALDI-TOF-MS to distinguish between related chemical structures, on the other hand, may be due to the mass (m/z) similarity of the flavonoids themselves. The use of bioautography TLC study and identification by MALDI-TOF allowed us to identify biologically active compounds existing in the plant extract, which have the ability to inhibit α-amylase.

The inhibitory activity of the flavonoids against α-amylase was compared, and the chemical structures of the flavonoids responsible for the inhibitory activity were investigated. According to our findings, formononetin has approximately 10 times the anti-α-amylase activity of apigenin. The activity of luteolin is comparable to that of apigenin. Genistein and kaempferol have a moderate level of activity.

The used technique of combining bioautography with MALDI-TOF-MS identification can be used for further study of plant components possessing anti-α-amylase activity. The results of quantitative determination of flavonoid inhibitory activity against α-amylase can serve for further development of a dietary supplement for the prevention of type 2 diabetes.

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Author contributions

Svetlana M. Filimonova: Formal analysis (equal); investigation (equal); validation (equal); writing – original draft (equal). **Anna Raysyan:** Investigation (equal); validation (lead); visualization (lead); writing – original draft (lead). **Jan O. Kaufmann:** Formal analysis (equal); software (equal); validation (equal); writing – review and editing (equal). **Evgeniy S. Melnikov:** Data curation (equal); investigation (equal); software (equal); validation (equal); visualization (equal); writing – original draft (equal). **Olga Yu. Shchepochkina:** Formal analysis (equal); writing – review and editing (equal). **Irina V. Gravel:** Project administration (equal); supervision (equal); writing – review and editing (equal). **Sergei A. Eremin:** Methodology (equal); project administration (equal); supervision (equal); writing – review and editing (equal).

Conflicts of interest

The authors declare that they have no conflict of interest.

Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ijfs.16491.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Chapter S1. TLC bioautography assay.

Table S2. Positive ion MALDI-TOF mass spectra of flavonoid standards.

Chapter S3. TLC determination of flavonoids.

Figure S4. TLC chromatogram under UV-light at 254 nm of flavonoids in the system n-hexane:ethyl acetate:glacial acetic acid (20:9:1, *v*/*v*/*v*).

Figure S5. TLC chromatogram under UV-light at 254 nm of the flavonoid kaempferol.

Figure S6. TLC chromatogram of extracts separated in the system n-hexan:ethyl acetate:glacial acetic acid (20:9:1, *v*/*v*/*v*).

Figure S7. TLC chromatogram under UV-light at 254 nm of extracts separated in the system n-hexane: ethyl acetate:glacial acetic acid (20:9:1, *v*/*v*/*v*).

Table S8. TLC-MALDI-TOF-MS analyses.

Chapter S9. Preparation of solution for determination of α-amylase inhibition activity of crude extracts and flavonoid aglycons.

Figure S10. TLC determination of compounds with anti-amylase activity in extracts *Lathyrus pratensis* L.

Chapter S11. Conditions of the LC-MS/MS analysis.

Table S12. Parameters of multiple reaction monitoring (MRM) MS/MS detection and retention times of the analytes.

Chapter S13. Sample preparation for LC-MS-MS.

Chapter S14. LC-MS-MS method validation for quantification of apigenin, genistein, luteolin, kaempferol, and formononetin.

Figure S15. Chromatogram of the pure solvent (A) and of 0.1 μg mL^{-1} level (b).

Table S16. Accuracy and precision of the method.

Chapter S17. Statistical analysis of the experiments.