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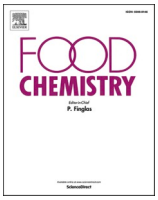

Quantification of mulberrin and morusin in mulberry and other food plants via stable isotope dilution analysis using LC-MS/MS

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ABSTRACT

Mulberry (*Morus*) is an important food and medicinal plant primarily used in sericulture. It is rich in prenylated flavonoids like morusin and mulberrin, which have shown promising bioactivities in vitro and in vivo but so far rely on HPLC methods for their quantification in plant material. Hence, a more sensitive LC-MS/MS stable isotope dilution assay for morusin and mulberrin was developed and validated. The analytes were quantified in 17 plant samples, with up to three plant compartments investigated (branches, leaves, fruits). The highest amounts were found in branches of a German mulberry tree (43 µg/g morusin, 910 µg/g mulberrin). Limits of detection reached 0.02 µg/g for morusin and 0.06 µg/g for mulberrin, which is significantly lower than existing methods, allowing for the detection and quantification of the analytes in leaves and fruits of mulberry, as well as common snowball (*Viburnum opulus*), a common food and medicine plant.

1. Introduction

Medicinal plants constitute a major source of pharmaceutical raw materials both for traditional as well as modern medicine systems (Bandaranayake, 2006). It is estimated that up to 80 % of the world's population relies on herbal medicinal products as a primary source of healthcare (Ekor, 2014). Often, medicinal plants are edible and serve a double purpose as nutraceuticals (Onaolapo & Onaolapo, 2022). Nutraceuticals are defined as "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease" (Brower, 1998). In recent years the commercialisation of nutraceuticals has strongly increased, reaching a market size of several hundred billion USD (Nwosu & Ubaoji, 2020).

Mulberry (*Morus*) is a multi-purpose agricultural plant, with an economic history of much over a thousand years (Sharma et al., 2000). Its primary usage is in sericulture, where mulberry leaves serve as food for silkworms, but it is also used for landscaping, timber, forage, food, and medicinal purposes. The largest crop areas of mulberry are in China (626,000 ha), and India (280,000 ha) (Sanchez, 2002). Hand in hand with its economic usage, mulberry farming produces considerable amounts of organic waste. Especially the branches from which the leaves were removed as well as stems and roots are byproducts of sericulture that are mostly discarded or used as firewood (Liu et al., 2010; Zhang et al., 2014). In China, the yearly amount of mulberry branches that enter waste streams is estimated to be 10 million tons (Ma et al., 2013). In mulberry fruit farming, significant amounts of leaves are an underutilized byproduct (Abdel-Khalek & Mattar, 2022). In light of increasing endeavours towards circular economy-oriented agricultural practices (Qi et al., 2016; Toop et al., 2017; Velasco-Muñoz et al., 2021), waste streams from mulberry farming are an attractive biological resource for the extraction of phytochemicals (Ma et al., 2013) and the production of nutraceuticals.

The health benefits of mulberry sources are diverse. In Chinese, Ayurvedic, and Unani traditional medicine (Jan et al., 2021), recipes from root epidermis, bark, or leaves are used to treat inflammations, fever, liver damage, and diabetes (Kadam et al., 2019; Sharma et al., 2020). Antioxidant (Majinda et al., 2011), antibacterial (Majinda et al., 2011), antiviral (Thabti et al., 2020), anti-inflammatory (Eo et al., 2014), anticancerogenic (Cheng et al., 2020; Eo et al., 2014) and hypoglycemic (Mudra et al., 2007) activities have been confirmed in modern scientific studies. Mulberry leaf and bark powder is widely used as a nutraceutical, including teas and infusions (Dhiman et al., 2020), as a dietary supplement (Dhiman et al., 2020; Lee et al., 2018; Panek-Krzyśko & Stompor-Gorący, 2021; Wang et al., 2018; Weng et al., 2021)

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or an additive to wheat flower (Khyade, 2020; Przeor & Flaczyk, 2016; Srivastava et al., 2003), even in toothpaste (Rivai et al., 2015). Fruit products include jam, jelly, yogurt, vinegar, molasses, and cosmetic creams (Dhiman et al., 2020; Yildiz, 2013).

Mulberry is known for being one of the few plant genera, that are rich in prenylated flavonoids (PFs) (Šmejkal, 2014). PFs are phytoalexins consisting of the characteristic flavonoid skeleton, substituted with a 3,3,-dimethylallyl (or related) group, that increases lipophilicity. In comparison to flavonoids, PFs have higher bioavailability and higher affinity to P-glycoprotein in biological membranes, making them promising drug candidates (Shi et al., 2021; Terao & Mukai, 2014; Yang et al., 2015). Morusin (MR) and mulberrin (MB) are among the most studied PFs in mulberry. Both have a flavone backbone with hydroxy-substitutions on positions 5, 7, 4′, and 6′ and prenyl groups at 3 and 8. In the case of MR, the 8-prenyl group is cyclized with the 7-hydroxy group, as shown in Fig. 1 (for numeration see Fig. S3 and S7 in supplementary information).

Both PFs have strong anti-oxidant and anti-inflammatory properties (Panek-Krzyśko & Stompor-Gorący, 2021; Zelová et al., 2014) and have shown anti-aging activity in vivo (Xu et al., 2023). MB has been shown to suppress Parkinson's disease abnormalities in vitro and in vivo (Cao et al., 2019). Moreover, it is frequently used in skin-whitening creams (Arung et al., 2006; Lee et al., 2004). MR has been highlighted for its therapeutic potential against diabetes (Choi et al., 2020), Alzheimer's disease (Borah et al., 2019; Choi et al., 2020; Zhu et al., 2022) and cancer (Hafeez et al., 2023; Kang et al., 2017; Lee et al., 2008; Lim et al., 2015; Wang et al., 2013), with promising in vivo evidence (Guo et al., 2016; Li et al., 2015) but lacking clinical trials.

Against this background, sensitive and accurate analytical methods for quantifying prenylated flavonoids like MR and MB in plant material are desired. Currently, the portfolio of quantification methods for MR and MB in plants is limited to HPLC methods with ultraviolet or diode array detection. To our knowledge, five studies on MR (Eom et al., 2017; Guo et al., 2020; Kim et al., 2015; Ma et al., 2013; Seo & Shin, 2018) and one on MB (Guo et al., 2020) quantification in plant material have been published, to date. While these HPLC-based methods are inexpensive and easy to operate, their low sensitivity limits their application to samples with high amounts of MR and MB. Most of the above studies focus on the quality control of medicinal products made from mulberry root. If the investigation of MR and MB is to be extended to plant compartments of mulberry that are less abundant in PFs, especially leaves, and fruits, the development of more sensitive methods is warranted. Especially nutraceuticals based on mulberry leaves or fruits will profit from analytical methods with higher sensitivity. Hence, the first aim of our study was to develop a mass spectrometry-based quantification method for MR and MB in plant samples that has lower limits of quantification (LOQ) and limits of detection (LOD) than existing HPLC-based methods. To the best of our knowledge, this would be the first validated LC-MS/MS method for quantifying MB, in general. For MR, so far, mass spectrometry methods have been developed exclusively in the pharmacological context with rat plasma or salt solution as matrices (Deng et al., 2017; Hou et al., 2018; Liu et al., 2019; Liu et al., 2021; Song et al., 2014). In the context of mulberry farming and nutraceutical production, complex matrices can arise, e.g. from agricultural waste streams or in the case of mixed herbal products that combine mulberry leaves, branches, and root bark (Eom et al., 2017). Our second objective was, therefore, to synthesize deuterium-labeled standards of MR and MB (MR-D and MB-D, see Fig. 1) and develop a stable isotope dilution assay for LC-MS/MS. Since the isotopically labeled standards almost entirely resemble the analytes in their physiochemical behaviour, they compensate for matrix influences, workup losses, and ionization interferences, making SIDA the "analytical gold standard" in mass spectrometry (Buckett et al., 2020; Rychlik & Asam, 2009). The usefulness of the method is demonstrated by analyzing MR and MB not only in mulberry branches, but also in leaves and fruits, as well as in other plant samples, for example common snowball (*Viburnum opulus*). Thus, the presence of these PFs in a variety of plants can be confirmed or negated.

2. Materials and methods

2.1. Chemicals and reference standards

All chemicals were received from commercial suppliers unless stated otherwise. Dimethylformamide (DMF) was purchased from Merck (Darmstadt, Germany), deuterated water (D_2O, 99.9 %) from Sigma-Aldrich (Steinheim, Germany), rhodium on alumina (Rh, 5 %) from Thermo Fischer Scientific (Waltham, USA), reference standards for MR and MB from Alpha Biotechnology (Chengdu, China).

2.2. Deuteration

All microwave reactions were carried out with a Discover SP-D microwave (CEM, Matthews, USA) in an 80 mL quartz microwave reaction flask with a magnetic stir bar (CEM). Deuterated standards for MR and MB were synthesized according to the following procedure: 7.70 mg of MR were put in a microwave reaction flask, dissolved in 1 mL DMF, and 10 mL D_2O and approx. 1 mg Rh (5 %)/alumina were added. The mixture was reacted in the microwave for 20 min at 150 °C and 21 bar with vigorous stirring. After the mixture had cooled, it was subjected to a three-time liquid extraction with ethyl acetate. The aqueous phase was discarded, and the organic phase was evaporated to dryness under reduced pressure. The residue was transferred into a fresh microwave vessel with 1 mL DMF, and the reaction was repeated (again with 10 mL D_2O, 1 mg Rh, for 20 min at 150 °C, 21 bar). The mixture was extracted as previously, and the reaction was repeated a third time to reach a satisfactory degree of deuteration. Similarly, MB was deuterated, with

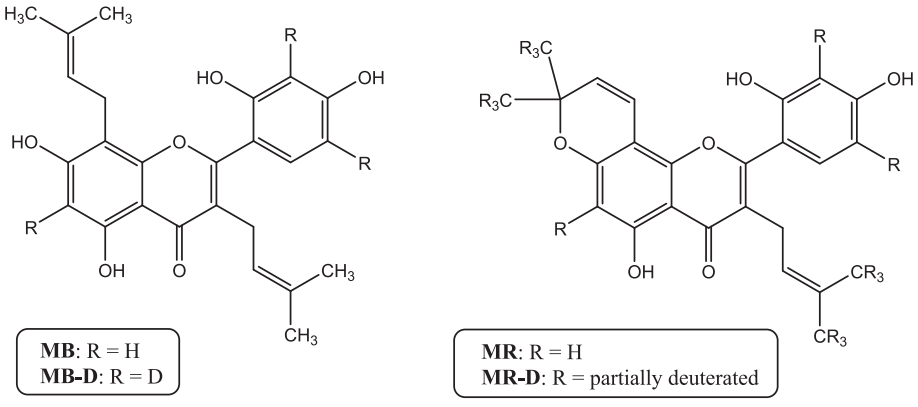

Fig. 1. Chemical structures of mulberrin (MB), deuterated mulberrin (MB-D), morusin (MR), and deuterated morusin (MR-D).

the only difference being that the reaction time was 60 min for the first two and 15 min for the third run. MR-D and MB-D were then purified separately using first a Besta (injection volume 0.5 mL) and then a Merck-Hitachi LaChrom preparative HPLC (injection volume 0.1 mL, conditions see below). Structure and degree of deuteration were checked using LC-MS/MS-qTOF and NMR, and concentration and purity were determined by analytical HPLC.

2.3. NMR

NMR experiments were performed in 500 µL acetone-d_6 (Sigma-Aldrich, St. Louis, USA) using NMR tubes (177.8 × 4.97 mm, Bruker, USA) on Bruker Avance III 400 MHz, 500 MHz, and 600 MHz NMR spectrometers by staff members at the Chair of Molecular Sensory Science at TUM. The chemical shifts are given in d-values (ppm) and the resonances of the solvent were used as internal shift reference. Coupling constants are given in Hz. The NMR spectra were evaluated using MestReNova software (Mestrelab Research, Spain).

2.4. Analytical and preparative HPLC

Analytical HPLC measurements were performed on an HPLC/DAD instrument (Shimadzu, Kyoto, Japan) with a DGU-20A3R degasser, LC-20AD liquid chromatograph, SIL-20AHT autosampler, and SPD-M20A diode array detector. Analytes were measured at their absorption maxima of 263 nm for MB and 269 nm for MR. A reversed phase column (YMC-Pack Pro C18, S-3 µm, 12 nm, 150 × 3 mm ID, precolumn: 8 × 3 mm, Machery-Nagel, Düren, Germany) was used. The column oven temperature was 40 °C. As solvents 0.1 % formic acid (FA) in ultrapure water (A) and 0.1 % FA in acetonitrile (ACN) (B) were used. The solvent gradient was run with a flow of 0.3 mL/min according to the following scheme: 50 % B (2 min isocratic), in 11 min to 90 % B, 90 % B (5 min isocratic), brought back to initial conditions in 1 min to 50 % B, 50 % B (3 min isocratic). Preparative HPLC was carried out on an HPLC BESTA system (Gastorr VG-25 Degasser, Besta Type HD 2–400 pump, Wilhelmsfeld, Germany, UV-VIS detector Sapphire Ecom, Prague, Czech Republic) with YMC-Actus Triart C18 column (S-5 µm, 12 nm, 150 × 20.0 mm ID, with C18 precolumn) and on a Merck-Hitachi LaChrom instrument with a L-7200 autosampler, L-7100 Pump, L-7400 UV-detector, D-7000 Interface and Degassex DG-4400 4-Channel On-line degasser (Phenomenex) with a C18 column (YMC-Pack Pro C18, 5 µm, 150 × 10 mm, P/N AS12S05-151OWT, YMC, Dinslaken, Germany). Preparative HPLC was performed at room temperature with the same solvent gradient as for analytical HPLC.

2.5. LC-MS-qTOF

Chromatographic separation was carried out on a Shimadzu Nexera X2 UHPLC system (Kyoto, Japan), with a FluoroPhenyl reversed-phase column (RaptorTM, FluoroPhenyl 2.7 µm, 100 × 2.1 mm) at a temperature of 40 °C. The solvent gradient composed of 0.1 % FA in ultrapure water (A) and 0.1 % FA in ACN (B) with a flow of 0.4 mL/min was run as follows: 10 % B (2 min isocratic), in 10 min to 70 % B, in 3 min to 85 % B, in 2 min to 98 % B, 98 % B (3 min isocratic), brought back to initial conditions in 1 min to 10 % B, 10 % B (4 min isocratic). Co-injection of 5 µL of sample together with 44 µL of water was used to approximate the chromatographic solvent composition at the start and, therefore, prevent pressure drops at injection. LOD samples were injected at 7.5 µL together with 42 µL of water. The solvent flow was introduced to a Shimadzu 9030 quadrupole time-of-flight (qTOF) mass spectrometer (TOF resolution: 30000 full width at half maximum, mass accuracy: <1 ppm). Measurements were carried out in negative electrospray ionization (ESI) and in multiple reaction monitoring (MRM) mode. The ion source parameters were as follows: Interface temperature 300 °C, heat block temperature 400 °C, desolvation line temperature 250 °C, interface voltage 4 kV, heating gas flow 10 L/min, drying gas flow 10 L/min, nebulizing gas flow 3 L/min, collision-induced dissociation gas pressure 230 kPa. Q1 transmission width was 5 *m/z*. MS/MS parameters were optimized by direct injection of the standard solutions (0.01 µg/mL to 1 µg/mL) into the ion source. The two most prominent mass transitions were used for the LC-MS/MS method. Data acquisition and data analysis were done with LabSolutions Software (Shimadzu, Kyoto, Japan). All final MRM parameters, collision energies, and retention times are listed in Table S1 in the supplementary information.

2.6. Storage stability tests

For the storage stability tests, MR and MB were stored in ACN at room temperature, at −27 °C, and at −80 °C over a time period of 165 days. At regular intervals, the samples were measured with analytical HPLC in order to observe changes in the chromatogram. Heat stability tests were carried out in ACN at 40 °C for four hours for both analytes separately.

2.7. Sample preparation

Plant samples were selected according to two criteria: being known to be rich in flavonoids or prenylated flavonoids, and being readily accessible to our laboratory. The following plant samples were examined: (1) *M. alba* branches GE ("white mulberry", from Germany), (2) *M. alba* branches SG1 (from Singapore), (3) *M. alba* branches SG2 (from Singapore), (4) *M. nigra* branches ("black mulberry", from La Palma, Spain), (5) *M. alba* leaves GE (from Germany), (6) *M. alba* leaves SG1 (from Singapore), (7) *M. alba* leaves SG2 (from Singapore), (8) *M. alba* fruit (from Turkey, commercially aquired), (9) *M. nigra* fruit (from Turkey, commercially aquired), (10) *Viburnum opulus* branches ("common snowball", from Germany), (11) *V. opulus* leaves (from Germany), (12) *V. opulus* fruits (from Germany), (13) *Robus idaeus* branches ("raspberry", from Germany), (14) *R. idaeus* leaves (form Germany), (15) *R. idaeus* fruits (from Germany), (16) *Durio zibethinus* fruit ("durian", chips, from Thailand, commercially acquired), (17) *Artocarpus heterophyllus* fruit ("jackfruit", from Singapore, commercially acquired). Additionally, *Crataegus monogyna* branches, leaves, and fruits ("common hawthorn", from Germany), which were known to be free from MR and MB, were prepared as matrices for method validation.

Harvested plant material was freeze-dried using an alpha 1–2 LDplus Martin Christ freeze dryer. Commercially obtained plant material had already been dried upon purchase. The dried plant material was then homogenized using a Grindomix GM 200 knife mill (Retsch, Germany) and if finer homogenization was needed a spice and coffee grinder (Rommelsbacher, Germany) or personal blender (Tribest, USA) was used. In order to prepare LC-MS/MS-qTOF samples, a 0.1 g aliquot of each plant material was taken and spiked with MR-D and MB-D as internal standard (IS). If the initial spiking amounts of IS resulted in a molar analyte/IS ratio out of the calibration range, the workup was repeated with an adapted amount of IS. After spiking, the samples were extracted with 1 mL MeOH for 45 min in an ultrasonic bath (VWR, Germany). The extracts were filtered through pleated filters, and the filters were rinsed with MeOH (4 × 1 mL). The filtrate was dried under nitrogen and subsequently reabsorbed in 1 mL MeOH and 2 mL aqueous NaOAc buffer (0.1 M, pH = 5, acidified with HCl). The samples were then subjected to solid phase extraction (SPE). For that, SPE cartridges (Chromabond, HR-X, 3 mL, 200 mg, Macherey-Nagel, Germany) were first pretreated successively with MeOH (3 mL) and NaOAc buffer (3 mL). The cartridges were then loaded with sample extract. Cartridge washing was performed with 3 mL of aqueous 5 % MeOH solution. Finally, the analytes were eluted from the cartridges with 6 mL of ACN: MeOH (50:50). The eluates were dried under nitrogen and washed into LC/MS vials with ACN (6 × 200 µL). The samples were then dried one more time under nitrogen and redissolved by vortexing in 1 mL of ACN. In order to prevent small solid particles from entering the LC-MS/MS system, all samples were centrifuged and the supernatant was pipetted

off and put into a fresh vial. Each sample was worked up three times and each workup was measured three times (total 9 measuring results per sample). All samples were stored at −27 °C in between uses.

2.8. Calibration and quantitation

In order to obtain calibration graphs for MR and MB, different amounts of analyte were mixed with constant amounts of IS in ratios of 0.013 to 80. The mixing process was done by autosampler co-injection and is given as an exact mixing scheme in Table S2 in the supplementary information. After LC-MS/MS measurements, calibration graphs were obtained by plotting the peak area ratios [A(A)/A(IS)] against the molar ratios [n(A)/n(IS)]. A fitting test according to Mandel (1964) was applied to determine the best-fitting regression model.

2.9. Method validation

Limits of detection (LODs) and limits of quantitation (LOQs) were determined according to Vogelgesang and Hädrich (1998). As an analyte free matrix, *C. monogyna* branches, leaves, and berries were used. 0.1 g matrix sample was spiked with analyte and IS, subjected to the workup procedure described above, and measured with LC-MS/MS. Each matrix sample was worked up in triplicate. In total, four spiking levels were prepared for each compound and matrix, reaching from approx. LOD to ten times LOD. The detailed spiking levels were as follows. Branches: 0.03, 0.10, 0.20, 0.30 µg/g MR; 0.10, 0.35, 0.70, 1.00 µg/g MB. Leaves: 0.08, 0.30, 0.60, 0.80 µg/g MR; 0.10, 0.35, 0.70, 1.00 µg/g MB. Berries: 0.04, 0.07, 0.10, 0.40 µg/g MR; 0.04, 0.07, 0.10, 0.40 µg/g MB. The IS was spiked in a constant amount of 0.35 µg/g for MR-D, and 0.08 µg/g for MB-D.

Injection precision was measured by injecting a sample mix containing both analytes and internal standards nine times one after the other. The concentrations were 5 µg/mL MR, 5 µg/mL MB, 1.1 µg/mL MR-D, and 1.5 µg/mL MB-D. For intra- and inter-day precision, sample 4 (*M. nigra* branches ESP) was worked up and measured nine times a day (intra-day precision, $n = 9$) on three different days (inter-day precision, $n = 3$). The precisions are given as coefficient of variation V_k with the formula $V_k(\%) = \frac{s}{\bar{x}} \bullet 100$ with s as standard deviation and $\bar{x}$ as sample mean.

Recoveries were prepared from *C. monogyna* branch matrix. Each recovery level was worked up twice and measured three times. The matrix was spiked with four different concentration levels (0.1, 15, 25, 50 µg/g for MR and 0.7, 200, 450, 900 µg/g for MB) in order to cover the concentration range of the samples. The recovery *rec* was calculated as the ratio of detected and spiked contents with the formula: $rec(\%) = \frac{n_{detected}}{n_{spiked}} \bullet 100$.

3. Results & discussion

3.1. Synthesis of isotopically labeled MR and MB

Since isotopically labeled standards for MR and MB are not commercially available, the deuterated products, as depicted in Fig. 1, were synthesized. Therefore, a microwave-based approach by Buckett et al. (2020) was adapted. For this, the flavonoids each were dissolved in DMF and then repeatedly incubated in the microwave together with deuterated water and catalyst. Contrary to Buckett et al., only rhodium on alumina was used as a single catalyst. Incubation temperatures and times as well as number of repetitions were optimized, balancing between product yield and deuteration degree. An overall yield of 30 % deuterated MB (thereof approx. 65 % D_3-MB) and 70 % deuterated MR (thereof approx. 25 % D_6-MR) was reached. The degree of deuteration was checked by LC-MS/MS-qTOF. As shown in Fig. S1 and S2 in the supplementary information, for both analytes no D_0-educt remained. D_1-product was not at all visible for MR, for MB only in negligible amounts (D_1 peak height 0.2 % of D_3). The absence of D_0-educt as well as natural isotopologues (like the D_1-product) is a requirement for the SIDA standard not to falsify the quantification (Rychlik & Asam, 2008). Fig. S1 shows that for MB, D_3-MB (*m/z* 424 $[M–H]^-$) was the main product and, therefore, chosen to be targeted in the SIDA method. Fig. S2 shows that for MR, a Gaussian curve-like distribution of the deuteration products was obtained, with D_5- and D_6-product (*m/z* 424 and 425 $[M–H]^-$) in approximately equal proportions as major products. Here, D_6-MR was chosen to be targeted because it showed a better coefficient of determination for the calibration function determined by the linearity test after Mandel (1964). For both D_3-MB and D_6-MR, NMR analysis gave indications on which positions were deuterated. As discussed in the supplementary information, Fig. S3–S6 and Table S3 display that in MB-D positions 6, 3′ and 5′ are deuterated. In MR-D, positions 6, 3′, and 5′, as well as the methyl groups are partially deuterated as outlined in Fig. S7–S10 and Table S4 in the supplementary information.

3.2. Optimization of sample preparation

Extraction times for the plant material (at the start of the workup) were optimized by testing sonication times between 10 min and 5 h. Plotting extraction efficiency over time, as depicted in Fig. S11 in the supplementary information, shows that an extraction time of 45 min serves as a suitable compromise between analyte extraction and time consumption. Since the workup involved multiple drying steps at 40 °C the heat stability of MR and MB was tested. The analytes were dissolved in ACN separately and incubated at 40 °C over 4 h, with aliquots being measured hourly by HPLC. As shown in Fig. S12 in the supplementary information, the peak areas of both analytes stayed constant at 100 ± 3 % and no formation of new peaks was observed. The respective drying steps, therefore, are unproblematic. Degradation of the analytes only took place over longer time intervals which was tested in storage stability tests. MR and MB were stored in ACN at room temperature, −27 °C, and − 80 °C for 165 days, with aliquots being measured regularly by HPLC. As depicted in Fig. S13 and S14 in the supplementary information, storage at −27 °C and − 80 °C showed no degradation of the analytes, but a linear decline of peak area was observed for the samples stored at room temperature, leading to a relative peak area of 84 % for MR and 75 % for MB after 165 days, compared to day 1. In order to avoid analyte degradation, all samples and standards were stored at −27 °C.

3.3. Validation

The generated calibration graphs underwent Mandel testing, which compared linear and quadratic regression models. The quadratic function was found to be more suitable for both analytes and both respective fragment ions (MR: *m/z* 297.1114 and 191.0697, MB: *m/z* 309.0403 and 299.1269, respectively). The heavier fragment ions showed better coefficients of determination and, therefore, were selected for calibration graphs. The molar ratio range was then selected to accurately cover the analyte/IS ratios obtained in the samples, which was about 1:15–10:1 for MR and 1:10–80:1 for MB. The calibration functions and coefficients of determination (R^2) are given in Table 1.

LODs and LOQs were obtained based on Vogelgesang and Hädrich (1998) by spiking an analyte-free matrix with MR and MB before the workup. As matrix, *C. monogyna* branches, berries, and leaves were used, which had previously been confirmed to be MR- and MB-free. The LODs and LOQs were calculated by a recovery function with four points ranging from approx. LOD to ten times LOD. All LODs and LOQs are summarized in Table 1. LODs and LOQs for different plant compartments varied with a factor of up to nine, showing the importance of matrix influence. The strongest matrix effects (therefore highest LODs) were observed for the leaf matrix, while, on the contrary, berries had the lowest LODs. Comparing these results to values from the literature is complicated. First, as mentioned previously, MR and MB in plant

Table 1
Calibration functions, LODs, LOQs, recoveries and precisions for MR and MB with the developed LC-MS/MS method.

		Morusin	Mulberrin
Calibration function		$y = -0.1984x^2 + 11.007x - 1.7491$	$y = -0.0061x^2 + 1.3852x + 0.8646$
R^2		0.9987	0.9961
Calibration range		n(MR)/(MR-D) = 0.067–11	n(MB)/(MB-D) = 0.11–79
LOD [μg/g]	Branches	0.05	0.16
	Leaves	0.18	0.27
	Berries	0.02	0.06
LOQ [μg/g]	Branches	0.13	0.65
	Leaves	0.63	1.25
	Berries	0.09	0.29
Recovery [%]	Level 1	89	104
	Level 2	84	79
	Level 3	103	74
	Level 4	72	58
Precision [%]	Inter-injection	2.78	4.33
	Intra-day	6.69	3.50
	Inter-day	10.2	6.26

samples have only been quantified by HPLC so far. Second, these studies have focused on mulberry root. For leaves and berries, to our knowledge, no LOD and LOQ data are available. Lowest reported method limits for HPLC analysis of mulberry root are LODs of 0.02 μg/mL for MR and 0.08 μg/mL for MB, and LOQs of 0.05 μg/mL for MR and 0.05 μg/mL for MB, reported by Guo et al. (2020). However, it is unclear how the LOD of MB can be higher than the LOQ, making these results seem unreliable. Since, third, that is the only study including MB, a discussion needs to focus on the more researched MR. Kim et al. (2015) published an LOD for MR of 0.069 μg/mL and LOQ of 0.229 μg/mL. Seo and Shin (2018) reached an LOD of 0.350 μg/mL and LOQ of 1.070 μg/mL for MR. All of these literature values were determined by a signal-to-noise ratio of 3 (LOD) and 10 (LOQ). The values of our study, which are given in μg/g in Table 1, can be converted into μg/mL by multiplying with 0.1 (since 0.1 g sample was weighed in and the final extraction solution was 1 mL). Our LODs and LOQs then underscore the literature values by a factor of at least 10, up to (in the case of Seo & Shin) a factor of 70. In general, the comparison of LOD and LOQ values with literature is cumbersome since not the same matrices were used and literature values were not obtained based on Vogelgesang and Hädrich (1998). It is clear, however, that the published HPLC methods would have had too high LODs and LOQs to detect and quantify the analytes in many of the samples that we investigated, especially the fruit samples of mulberry and common snowball. While HPLC methods are suitable for quality control of mulberry wood samples, other plant compartments (with lower concentrations) require the sensitivity of an LC-MS/MS method. A useful application would be the quantification of MR and MB in capsules filled with mulberry leaf and fruit powder, which are commercially available. With slight adaptions to the workup, the high sensitivity of the SIDA could also be employed in the analysis of MR and MB in blood or urine samples, e.g. in pharmacokinetic or metabolic pathway studies.

Recoveries and precisions are summarized in Table 1. Recoveries lay in the acceptable range of 70–120 % (according to European Commission, 2018 SANTE/11813/2017), except the highest concentration level of MB. Here, the recovery was 58 %, which is due to a high molar analyte/IS ratio of 65. For samples in that concentration range (in this study only *M. alba* branches GE) it is advised that only half the amount of sample (50 mg) is used for analysis. Inter-injection, intra-day, and inter-day precision lay in a range of around 3 to 10 %, which is well below the recommended ≤20 % (European Commission (2018) SANTE/11813/2017).

3.4. Contents of MR and MB in 17 different plant samples

The developed SIDA method was used to quantify MR and MB in 17 different plant samples. The samples were from different plant compartments of white mulberry (*M. alba*), black mulberry (*M. nigra*), common snowball (*V. opulus*), raspberry, (*R. idaeus*), durian (*D. zibethinus*), and jackfruit (*A. heterophyllus*). Since mulberry is known to contain both analytes, the study focused on this plant more than the others, analyzing three different plant compartments (branch, leaf, fruit) of different trees. Fig. 2 shows the LC-MS/MS chromatogram of an exemplary mulberry sample, *M. alba* branches SG1.

For identifying a peak as MR or MB, three identification criteria were applied: Retention time identity with the IS, presence of the two major mass transitions (compared to a purchased analyte standard), and constant ion intensity ratio, i.e. area of highest mass transition divided through area of second highest mass transition, compared to the IS and a purchased analyte standard. Further LC-MS/MS chromatograms of a standard mix without matrix (Fig. S15) and a quality control (Fig. S16) are given in the supplementary information.

Beyond mulberry, the other selected plants are known to be rich in flavonoids or prenylated flavonoids (Akazawa et al., 2020; Aziz & Jalil, 2019; Bradish et al., 2012; Goławska et al., 2023; Kajszczak et al., 2020; Staszowska-Karkut & Materska, 2020; Ye et al., 2019) and were chosen to investigate the presence of MR and MB with the aim of finding new sources outside of mulberry. All quantification results for MR and MB are summarized in Table 2.

As listed in Table 2, MR and MB were mainly found in mulberry samples. Within different plant compartments of mulberry, it is known that MR is mostly present in the wooden parts (root, branch), and significantly less in leaves and fruits (Ma et al., 2013). This distribution can be confirmed with our data. The highest amounts of MR were found in two branch samples, *M. alba* GE and *M. alba* SG1 with 43 and 40 μg/g, respectively. Only the leaves of *M. alba* GE contained MR, albeit in amounts below LOQ. Black mulberry fruits contained 0.15 μg/g MR. No MR was found in white mulberry fruits. For MB, *M. alba* branches GE contained a significant amount of 910 μg/g MB, the highest in the study. This was also the sample with the highest MR content. The leaves of that tree had a surprisingly high MB content of 20 μg/g, surpassing even most branch samples of other trees. Vice versa, these leaves hardly contained any MR. Apart from a few exceptions, there are two general trends: MB contents are higher than MR contents, and samples that score high in one of the analytes also have high amounts of the other. In fact, if the samples are arranged in order of the highest contents, the sample ranking looks similar for both MR (in decreasing content order samples no. 1, 2, 4, 3, 9) and MB (1, 2, 5, 4, 3, 9). Both trends indicate that MB is likely the biosynthetic precursor of MR, given that cyclisation with a neighbouring hydroxyl group to form a 2,2-dimethyl-pyran belongs to the most common *in planta*-modifications of the prenyl group (Šmejkal, 2014).

Besides mulberry, the study also analysed MR and MB in leaves and fruits of common snowball (*V. opulus*). Snowball leaves contained MB in detectable amounts, but below LOQ. Snowball fruits contained MR levels below LOQ. An MB content of 0.5 μg/g could be quantified in snowball fruits, which is similar to the amount of MB in mulberry fruits. The branches did not contain the analytes. It is rare that MR and MB are found in plants outside the family *Moraceae*. Besides *Morus*, the most common sources of MR and MB are in the genera *Artocarpus* (Arung et al., 2006, 2011; Arung et al., 2010; Jin et al., 2015; Ma et al., 2010; Panthong et al., 2013; Shamaun et al., 2010; Soekamto et al., 2003; Suhartati et al., 2013; Toume et al., 2015; Zhao et al., 2009) and *Maclura* (Ko et al., 2020; Nomura et al., 1990; Quang et al., 2015), both (like *Morus*) belonging to *Moraceae*. Other reports on MR and MB are scarce and scattered (Choi et al., 2022; Hu et al., 2021; Jing et al., 2019; Kapche et al., 2017; McPherson et al., 2022; Salunke et al., 2022; Sun et al., 2016), which might be in part due to (so far) insensitive analytical methods. Common snowball is widespread in Europe, western, and

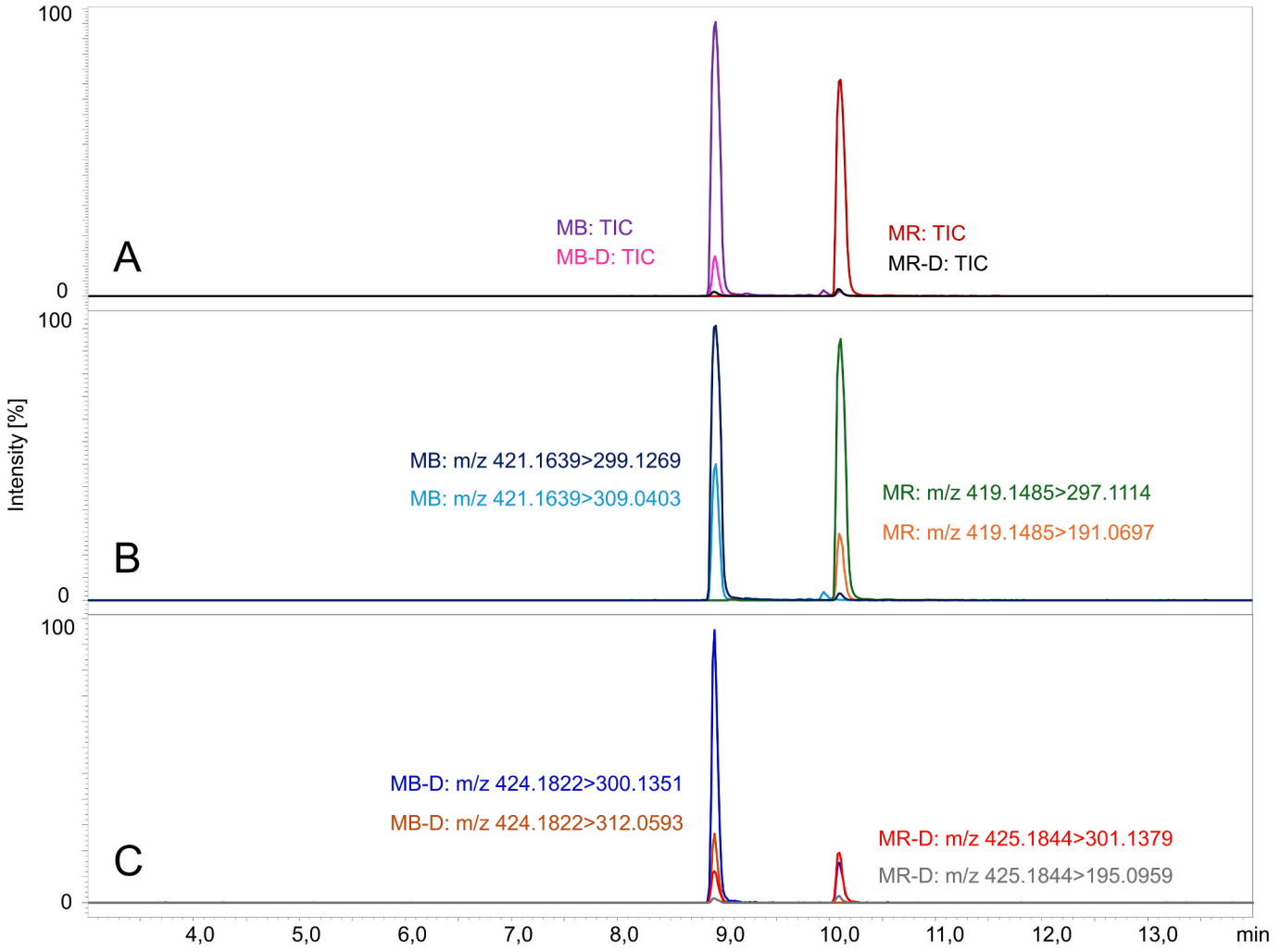

Fig. 2. LC-MS/MS chromatogram of *M. alba* branches SG1. **A**: Total ion chromatograms (TIC) of MB, MB-D, MR, and MR-D. **B**: Extracted ion chromatograms (EIC) of MB and MR. **C**: EICs of MB-D and MR-D.

Table 2
Contents of MR and MB in 17 different plant samples ($n = 9$). Values below LOQ and LOD are marked as "n.q." and "n.d.", respectively.

No.	Sample	Morusin [μg/g]	Mulberrin [μg/g]
1	*M. alba* branches GE	43.2 ± 2.4	910 ± 80
2	*M. alba* branches SG1	39.6 ± 4.3	79.9 ± 7.7
3	*M. alba* branches SG2	0.34 ± 0.04	1.7 ± 0.1
4	*M. nigra* branches ESP	0.92 ± 0.06	3.3 ± 0.4
5	*M. alba* leaves GE	n.q.	20.2 ± 6.5
6	*M. alba* leaves SG1	n.d.	n.q.
7	*M. alba* leaves SG2	n.d.	n.d.
8	*M. alba* fruit	n.d.	0.54 ± 0.22
9	*M. nigra* fruit	0.15 ± 0.01	0.65 ± 0.19
10	*V. opulus* branches	n.d.	n.d.
11	*V. opulus* leaves	n.d.	n.q.
12	*V. opulus* fruits	n.q.	0.50 ± 0.04
13	*R. idaeus* branches	n.d.	n.d.
14	*R. idaeus* leaves	n.d.	n.d.
15	*R. idaeus* fruits	n.d.	n.d.
16	*D. zibethinus* fruit	n.d.	n.d.
17	*A. heterophyllus* fruit	n.d.	n.d.

northern Asia and is used in a wide variety of culinary contexts (Česonienė et al., 2010). Snowball fruits are common in Russian, Ukrainian, and Siberian cuisine as ingredients in sauces, cakes, and drinks (Česonienė et al., 2010). In Scandinavia, snowball fruits are cooked into preserves, in Canada they substitute for cranberries (Velioglu et al., 2006). Snowball-based drinks are especially popular in Turkey, including juice, tea, cordials, liqueurs, and fermented drinks (Zakłos-Szyda et al., 2020). Snowball fruit juice is, in fact, a suitable object for future MR and MB quantification studies to test if the juicing process enriches the PFs of the berries. Besides its culinary use, snowball is well-known for its nutraceutical properties. Snowball fruits have been applied in folk medicine to treat a wide range of illnesses, including heart disease, coughs and colds, tuberculosis, shortness of breath, digestive problems, as well as duodenal ulcers and bleedings (Česonienė et al., 2012; Velioglu et al., 2006). Modern scientific studies on snowball fruit juice confirmed antioxidant (Zakłos-Szyda et al., 2020), anti-inflammatory (Česonienė et al., 2010), antimicrobial (Česonienė et al., 2012; Česonienė et al., 2014), anti-tumor (Ceylan et al., 2018) and anti-cancer (Gok et al., 2023; Stępień et al., 2018; Zakłos-Szyda & Pawlik, 2019) properties, as well as preventive potential for metabolic diseases (Česonienė et al., 2010; Zakłos-Szyda et al., 2020). Because of its "astringent-bitter-sour-taste" (Kajszczak et al., 2020), research is carried out to remove astringent taste compounds and further promote the commercialisation of common snowball juice (Velioglu et al., 2006). Like mulberry, snowball is a versatile food and medicine plant with high potential for the development of nutraceuticals. Sensitive methods for PF analysis, like the developed SIDA, can accompany this development. In all other plant samples (*R. idaeus*, *D. zibethinus*, and *A. heterophyllus*) no MR and MB could be detected.

The most comprehensive study on MR in plant samples has been

undertaken by Ma et al. (2013), who quantified MR in the branch bark of 20 different mulberry trees. Their values ranged between 37 and 314 μg/g, with even two samples from the same species (*M. alba*) showing a difference of 119 versus 214 μg/g MR. The values in our study lie at the lower end of this spectrum. Our results confirm the strong variations in MR and MB content within mulberry trees of the same species, which is especially evident in the case of two Singaporean white mulberry trees that grew under similar conditions (samples 2 and 3). A more widespread study on climatic and regional influences, as well as tree-to-tree and intra-tree distributions, can elucidate factors that promote (or inhibit) the biosynthesis of MR and MB. SIDA methods are especially suited for such investigations, because matrix influences, ionization interferences, and workup losses are compensated for by the isotopologic standard, making comparisons between different samples as reliable as possible. The reported deuteration method can easily be extended to other PFs, making the presented method an optimal choice for the quantification of pharmacologically relevant PFs in organic waste streams from mulberry farming or new nutraceutical products.

4. Conclusion

Despite its phytochemical potential mulberry is still struggling to "find its identity" in the food and medicine market and remains underutilized (Dhiman et al., 2020). The current trend towards nutraceuticals is a chance to rediscover this ancient, culturally important plant in an age of increasingly industrialized, standardized, and commercialized food and medicine production. The reported SIDA is a necessary chaperone for this development. The low LODs and LOQs promote the analysis of MR and MB in nutraceutical products, e.g. capsules with mulberry leaf or fruit powder, sold as dietary supplements, or common snowball fruit juices. The stable isotope approach is especially useful for complex matrices, like recently developed mixed herbal products that combine mulberry leaves, branches, and root bark (Eom et al., 2017), or agricultural waste streams from mulberry farming whose PF content is of interest for the pharmaceutical industry (Ma et al., 2013; Zhang et al., 2014). The high sensitivity of the method allowed for the analysis of MR and MB in common snowball, when it is typically rare to find the analytes outside of mulberry. This would not have been possible with existing HPLC-based methods. Existing knowledge on the order of magnitude, distribution, and variability of MR contents in mulberry was confirmed in this study. A similar picture emerges for the less researched MB, with high variations between samples of similar origin (e.g. *M. alba* branches SG1 and SG2). Two prospects might constitute the next steps in this field of research. In-depth studies on which factors promote (or hinder) the biosynthesis of PFs in mulberry. And the production of PFs by metabolic engineering of which the first steps have already been taken (Isogai et al., 2021; Levisson et al., 2019; Yang et al., 2015). In both cases, the developed SIDA can serve as a sensitive and accurate quantification method, not only for MR and MB but, since the proposed microwave-assisted deuteration can be readily adapted, for PFs, in general.

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CRediT authorship contribution statement

Christian Schnurr: Writing – original draft, Validation, Methodology, Investigation, Data curation. **Lance Buckett:** Writing – review & editing, Validation, Supervision, Methodology, Investigation. **Jana Bitenc:** Validation, Methodology, Investigation. **Michael Rychlik:** Writing – review & editing, Supervision, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.143061.

Data availability

Data will be made available on request.

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