**DETERMINATION OF D-FAGOMINE IN BUCKWHEAT AND
MULBERRY BY CATION EXCHANGE HPLC/ESI-Q-MS**

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Running title. D-Fagomine and diastereomers in buckwheat and mulberry.

ABSTRACT

D-Fagomine is an iminosugar first found in buckwheat (*Fagopyrum sculentum* Moench) which if used as a dietary supplement or functional food component may reduce the risks of developing insulin resistance, becoming overweight and suffering from an excess of potentially pathogenic bacteria. As D-fagomine may become increasingly important to the food industry, a reliable analytical method for its determination in natural plant sources and foodstuffs is desirable. We have devised a method to separate D-fagomine from its diastereomers 3-*epi*-fagomine and 3,4-di-*epi*-fagomine in a single run by cation exchange high performance liquid chromatography with detection and quantification by mass spectrometry using electrospray ionisation and a simple quadrupole analyser (ESI-Q-MS). The method is validated and applied to the determination of D-fagomine in buckwheat groats (6.7-44 mg kg⁻¹), leaves, bran and flour. We show that buckwheat contains 3,4-di-*epi*-fagomine (1.0-43 mg kg⁻¹), which has not previously been reported in this source. The procedure is also applied to mulberry (*Morus alba*) leaves, which contain D-fagomine and 3-*epi*-fagomine as minor components. The new method provides a means for convenient and accurate determination of D-fagomine in plant samples and foodstuffs.

Keywords. D-fagomine, iminosugars, iminocyclitols, buckwheat, mulberry, HPLC/MS.

1 Introduction

2

3 D-Fagomine (Fig. 1) is an iminocyclitol first isolated from seeds of buckwheat (*Fagopyrum*

4 *sculentum* Moench, Polygonaceae) [1] and later found in other plant sources [2].

5 Iminocyclitols, also referred to as iminosugars, are carbohydrate analogues in which the

6 endocyclic oxygen has been replaced by nitrogen and which have spatial configurations of the

7 hydroxyl groups that coincide with those of sugars (e.g. glucose, galactose, fucose, and

8 mannose). As D-fagomine and other iminosugars such as 1-deoxynojirimycin (DNJ) are

9 intestinal glycosidase inhibitors, they have the potential to lower post-prandial blood glucose

10 concentration. Thus they can reduce the risk of developing insulin resistance and becoming

11 overweight[3]. Moreover, natural and chemically modified iminocyclitols present other

12 activities which may help prevent disease or have therapeutic applications [4,5]. We have

13 recently shown that D-fagomine exerts a double action during its transit along the intestinal

14 tract. First, it effectively delays the breakdown of sucrose and starch, thus lowering blood

15 glucose concentration without affecting insulinemia; and second, it selectively agglutinates

16 fimbriated *Enterobacteriaceae* such as *Escherichia coli* and *Salmonella enterica* and

17 promotes the adhesion of probiotic bacteria to the intestinal mucosa [6]. As a minor

18 component of buckwheat, D-fagomine is present in many traditional foodstuffs from around

19 the world. These include Japanese soba noodles, French crêpes de Bretagne, Italian polenta,

20 Slavic blinis, Spanish farinetes de fajol from Catalonia and a myriad of others. Its history of

21 human consumption together with its bioactive properties makes D-fagomine a suitable

22 candidate to become a dietary supplement and/or functional food component.

23 Since iminosugars lack chromophores, their detection and determination in natural sources

24 and foodstuffs cannot usually be performed using spectrophotometric techniques.

25 Derivatization of the analytes may produce species that can be analysed by liquid

1 chromatography coupled to a fluorescence detector (LC-FLD) [7,8]. Derivatization may also
2 be used to obtain volatile derivatives that can be analysed by gas chromatography (GC-FID or
3 GC-MS) [9-14]. However, the efficiency of the derivatization and the stability of the resulting
4 compounds are crucial factors that must be accurately known and carefully controlled.
5 Iminosugars are usually analysed through purification followed by characterization by mass
6 spectrometry (MS) and/or nuclear magnetic resonance (NMR) [15-17]. Tedious prior
7 separation of the components may be avoided if efficient LC stationary phases are found that
8 can be coupled to MS. Due to their high polarity and cationic nature, iminocyclitols can be
9 separated by hydrophilic interaction (HILIC) on amide resins and ionized by thermo spray
10 (TSI), electrospray (ESI) or atmospheric pressure chemical ionization (APCI) [18-20] prior to
11 MS detection. Light scattering detection (LSD) has also been used [18] but it is less selective
12 and less sensitive than MS.

13 Using different combinations of chromatography and spectrometry, the iminosugar D-
14 fagomine has been detected and quantified in *Morus bombycis* leaves (Moraceae) [21],
15 mulberry (*Morus alba*) root bark, fruit and leaves (Moraceae) [17,20,22], *Lycium chinense*
16 roots (Solanaceae) [23], *Xanthocercis zambesiaca* roots and leaves (Fabaceae) [15],
17 *Angylocalyx pinaertii* pods (Fabaceae) [24] and *Baphia nitida* leaves (Fabaceae) [25], as well
18 as buckwheat (*Fagopyrum sculentum*) [14,26] where it is the only iminosugar to have been
19 found. The roots of *Xanthocercis zambesiaca* contain the highest amount of D-fagomine
20 reported to date (1.3 g kg⁻¹). D-Fagomine has also been detected in *Castanospermum australe*
21 seeds (Fabaceae) [16], *Lonchocarpus latifolius* roots (Fabaceae) [12], *Morus multicaulis*
22 leaves (Moraceae) [27] and *Morus atropurpurea* root bark (Moraceae) [28]. Some of these
23 sources also contain diastereomers of D-fagomine, specifically 3-*epi*-fagomine and 3,4-di-*epi*-
24 fagomine (Fig. 1). As the activity of D-fagomine and of its diastereomers may not be the
25 same, it is important to determine the diastereomeric composition in natural sources. 3-*epi*-

Fagomine has been determined in *Xanthocercis zambesiaca* [15], mulberry [17] and *Baphia nitida* [25], while 3,4-di-*epi*-fagomine has been determined in *Xanthocercis zambesiaca* [15]. D-Fagomine, 3-*epi*-fagomine and/or 3,4-di-*epi*-fagomine from *Xanthocercis zambesiaca* and mulberry have been isolated using consecutive anionic and cationic resins [15,17]. Chen *et al.* [16] reported the presence of two D-fagomine diastereomers and three putative deoxy compounds in *Castanospermum australe* seeds. A first fractionation using a silica-based amino column separated D-fagomine from the two unidentified stereoisomers that were then analysed by cation exchange HPLC. None of the procedures in the literature provides a separation of D-fagomine and its diastereomers in a single analytical run and no stereoisomers of D-fagomine have been found in buckwheat to date.

The aim of this paper is to provide a fast, sensitive and selective LC/MS method for the analysis of D-fagomine in natural sources and foodstuffs. The method thus developed is successfully applied to the quantitative analysis of D-fagomine in buckwheat leaves, bran, groats and flour as well as in mulberry leaves.

Materials and Methods

Samples

Buckwheat (*Fagopyrum sculentum*) groats were obtained from Can Rovira (Batet de la Selva, Spain) (brand A) and BioSpirit (Girona, Spain) (brand B). Buckwheat (*Fagopyrum tataricum*) was obtained from the Botanischer Garten and Botanisches Museum Berlin-Dahlem (Berlin, Germany) (brand C). Buckwheat (*Fagopyrum sculentum*) flour was from Can Rovira (brand A) and from El Granero Integral (Madrid, Spain) (brand D); buckwheat bran was supplied by Can Rovira (brand A) and by the Botanischer Garten and Botanisches Museum Berlin-

Dahlem (brand C); while buckwheat leaves were also from Can Rovira. Mulberry leaves were collected in a local groove. The samples were stored at room temperature in hermetically sealed containers until analysis; except for the leaves, which were kept in hermetically sealed bags at -20°C .

Reagents

The D-fagomine standard was synthesized in the laboratory following a published and patented enzymatic method [29,30]. A mixture of D-fagomine, 3-*epi*-fagomine and 3,4-di-*epi*-fagomine (or its enantiomer 5-*epi*-fagomine) was obtained as a partially purified product in the synthesis of D-fagomine. DMDP (2,5-hydroxymethyl-3,4-dihydroxypyrrolidine), the internal standard, was purchased from IRL (Lower Hutt, New Zealand). Lichrosolv grade methanol, and analytical grade acetic acid and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). Synthesis grade hexane (alkanes mixture), ethanol and sodium hydroxide were purchased from Panreac (Barcelona, Spain). Millipore type I water was used to prepare all of the aqueous solutions. Solid-phase extraction (SPE) cartridges for sample purification were Spe-ed, SCX (strong cation exchange) cartridges, 100 mg mL^{-1} from Applied Separations (Allentown, PA, USA). Nylon filters ($0.45\text{ }\mu\text{m}$) were obtained from Phenomenex (Torrance, CA, USA). IRC86SB and IRA458CL resins were from Rohm and Hass (Chauny, France). CM Sepharose Fast Flow resin was obtained from GE Healthcare (Uppsala, Sweden).

Extraction and SPE cleanup

The method used for the extraction of D-fagomine and its diastereomers depended on the nature of the samples. Groats and bran were milled using a Moulinex A 505 2HF mill (Ecully Cedex, France) while leaves were crushed in a mortar with liquid N₂. Then the milled samples (100 mg) were spiked with 70 µL of a methanolic solution containing 100 mg L⁻¹ DMDP covered with perforated parafilm and left overnight until complete evaporation of the solvent. D-Fagomine was extracted with 70% aqueous methanol (12 mL) using an Intelli-mixer RM-2 orbital shaker (Elmi; Riga, Latvia) for 15 min. The extracts were centrifuged, filtered using a 0.45 µm nylon filter and the filtrates were loaded onto SCX cartridges, previously conditioned with methanol (1 mL) and water (1 mL). After loading, the SCX resin was washed with water (3 mL) and vacuum dried. Then D-fagomine and its diastereomers were eluted with 2 M aqueous NH₄OH (450 µL). The eluates were evaporated to dryness under a stream of nitrogen at 60°C and the residues were redissolved in water (400 µL) and passed through a 0.45µm nylon filter. The samples were stored at 4°C and analysed within 48 h after preparation.

HPLC/ESI-Q-MS analysis

Chromatography was carried out on an Agilent 1100 system (Santa Clara, CA, USA) equipped with a binary pump and fitted with either a TSK-Gel Amide 80 hydrophilic interaction column (15 cm × 2 mm i.d., 3 µm particle size) (Tosoh Bioscience; Tokyo, Japan) or a TSK-Gel CM2SW cation exchange column (25 cm × 4.6 mm i.d., 5 µm particle size) (Tosoh Bioscience) preceded by the corresponding guard cartridge (1 cm). The injection volume was 20 µL and the column temperature 25°C. The target compounds were separated with a binary system consisting of aqueous NH₄Ac and acetonitrile or methanol. MS analysis of the column effluent was performed on an ESI/TSQ 7000 mass spectrometer (Thermo

Scientific; West Palm Beach, FL, USA). The final source parameters were as follows: voltage, 4.5 kV; capillary temperature, 250°C; sheath gas, 60 psi; auxiliary gas, 30 psi; and multiplier voltage, 1350 V. Analyses were carried out in the selected ion monitoring (SIM) mode and quantitative measurements were achieved using DMDP as the internal standard. $[M + H]^+$ ions at m/z 148.2 (D-fagomine and diastereomers) and 164.2 (DMDP) were monitored in the first quadrupole.

The exact masses of D-fagomine and its diastereomers were obtained using high resolution ESI-TOF-MS on an LCT Premier XE system (Waters; Milford, MA, USA) after high resolution separation using an HPLC Acquity system (Waters) fitted with a TSK-Gel CM2SW column. From buckwheat groats: D-fagomine, t_R 14 min, m/z found 148.0974 (calculated for $C_6H_{14}O_3N$ 148.0975, 0.7 ppm); 3,4-di-*epi*-fagomine, t_R 24 min, m/z found 148.0974 (calculated for $C_6H_{14}O_3N$ 148.0975, 0.7 ppm). From mulberry leaves: D-fagomine, t_R 15 min, m/z found 148.0968 (calculated for $C_6H_{14}O_3N$ 148.0975, 4.1 ppm); 3-*epi*-fagomine, t_R 22 min, m/z found 148.0969 (calculated for $C_6H_{14}O_3N$ 148.0975, 3.4 ppm).

Purification and characterization of 3,4-di-*epi*-fagomine from buckwheat groats

Milled buckwheat groats (420 g) were defatted with hexane (3 x 1.5 L) and the remaining solid (400 g) was stirred with 70% aqueous ethanol (8 L) for 45 min. Then the mixture was filtered and an aliquot of the filtrate (4 L) loaded onto a column packed with IRC86SB resin (cation exchange, 600 mL bed volume). After washing with water (1 bed volume) and 0.04 M aqueous ammonia (1.5 bed volumes), the iminosugar fraction was desorbed with 0.15 M aqueous ammonia (2 bed volumes). The ammonia was eliminated from the eluate by evaporation and the solution passed through a column packed with IRA458CL resin (anion

exchange, 400 mL bed volume). After washing with water (1 bed volume) the non-retained eluate contained the iminosugars. This fractionation procedure was repeated with the other aliquot (4 L) of the filtered buckwheat extraction mixture. The two eluates from anion-exchange were pooled and freeze-dried. This solid was dissolved in water (4 mL), the pH adjusted to 6.5 with acetic acid and the solution passed through a 0.45 μ m nylon filter and loaded onto a column packed with CM Sepharose Fast Flow resin (220 mL) fitted to an FPLC system (Pharmacia Biotech, Uppsala, Sweden) previously conditioned with 0.5 M aqueous ammonia (2 bed volumes) and water (3 bed volumes) at 4 mL min⁻¹. The resin was washed with water (2 bed volumes) and the iminosugars fractionated with 0.01 M aqueous ammonia (5 bed volumes) at 3 mL min⁻¹. Fractions (110 x 7.5 mL each) were collected and analysed by HPLC/ESI-Q-MS under the conditions described above. D-Fagomine was detected in fractions 30-40, while the putative 3,4-di-*epi*-fagomine was detected in fractions 90-110. Fractions 90-110 were pooled, freeze-dried and subjected to NMR on an Avance II Plus 600 NMR system from Bruker (Rheinstetten, Germany). ¹H NMR [(D₂O, 600 MHz): δ 3.87 (dd, J = 7.9 Hz, 4.4 Hz, 1H, H₃), δ 3.71 (dd, J = 4.7 Hz, 2.3 Hz, 1H, H₄), δ 3.64 (m, 2H, H₆), δ 3.16 (ddd, J = 7.6 Hz, 5.6 Hz, 2.1 Hz, 1H, H₅), δ 2.91 (m, 2H, H₁), δ 1.97 (m, 1H, H₂), δ 1.58 (ddd, J = 14.7 Hz, 7.6 Hz, 3.5 Hz, 1H, H_{2'}). The chemical shifts were consistent with the structure of 3,4-di-*epi*-fagomine or its enantiomer, 5-*epi*-fagomine. The NMR spectra of the purified diastereomer and a synthetic standard are included as Electronic Supplementary Material Figs. S1 and S2.

Standard solutions

A stock standard solution of 20 mg L⁻¹ was prepared by dissolving D-fagomine (1.00 mg) in methanol (50 mL). A stock internal standard solution of 100 mg L⁻¹ was prepared by dissolving DMDP (1.00 mg) in methanol (10 mL). Both solutions were stored at -20°C. To prepare the working standard solutions, the corresponding aliquot of D-fagomine stock solution was mixed with 70 µL of the DMDP stock solution. Then the solvent was evaporated to dryness under a stream of N₂ and the residue dissolved in 400 µL of water. These solutions were also stored at -20°C. The standard solutions were stable at this temperature for a period of at least 6 months. Stability was tested by comparing the peak areas of the standards with that of freshly prepared solutions of D-fagomine using the HPLC/ESI-Q-MS conditions described above.

Validation of the analytical method

Cation exchange chromatography on a TSK-Gel CM2SW column was chosen as the separation step after SPE clean-up and prior to MS detection. The linear MS responses of D-fagomine and DMDP dissolved in pure water and matrix-matched solutions were compared. Matrix-matched solutions were obtained by extracting the corresponding matrix as described above. Calibration curves were constructed by plotting A_{D-fagomine} (the D-fagomine area) against the D-fagomine concentration and A_{D-fagomine}/A_{DMDP} against the D-fagomine concentration for each solvent. The solutions were prepared and analysed in triplicate. The signal suppression/enhancement (SSE) due to matrix effects was calculated as follows:

$$\text{SSE (\%)} = \frac{\text{Slope}_{\text{matrix-matched standard}}}{\text{Slope}_{\text{aqueous standard}}} \times 100$$

In the assessment of linearity, a calibration curve was plotted in the range 1-10 mg L⁻¹ (4-40 mg kg⁻¹). Five calibration standards were analysed across this range. All of the calibration

standards were spiked with DMDP standard stock solution (70 μL) (17.5 mg L^{-1}). Linearity was checked each day of the study to compensate for ESI variability.

A precision and recovery study was also carried out with buckwheat groats. The European Medicines Agency (EMA) [31] recommends a recovery value (accuracy) in the range 85%-115% and a relative standard deviation value (RSD, precision) under 15% for bioanalytical methods. The precision and recovery of the method were studied by spiking milled buckwheat groats with D-fagomine at concentrations of 10, 20 and 40 mg kg^{-1} , in triplicate. The samples were processed 24 h after spiking, to ensure complete evaporation of the solvent. After elution in the purification step, the samples were spiked with the DMDP standard stock solution (70 μL) to correct for ESI variability. Recovery was determined by comparing the $A_{\text{D-fagomine}}/A_{\text{DMDP}}$ signal obtained from the spiked buckwheat samples with the $A_{\text{D-fagomine}}/A_{\text{DMDP}}$ signal from the calibration standards. The D-fagomine content in non-spiked material was determined and subtracted from the value obtained for the spiked samples. The limit of quantification (LOQ) was established as the concentration corresponding to 10-times the signal-to-noise ratio calculated using the Xcalibur[®] software from Thermo Scientific.

Results and discussion

HILIC/ESI-Q-MS separation of D-fagomine and internal standard

With the final aim of quantitatively analysing D-fagomine in natural sources by HPLC/ESI-Q-MS, a hydrophilic interaction chromatography (HILIC) TSK-Gel Amide 80 column was first selected, in accordance with the literature [18-20]. The behaviour of both standard D-fagomine and the internal standard, DMDP, was tested under different elution conditions. The mobile phase components were acetonitrile and aqueous NH_4Ac . Gradients from 15%-26% to

30%-70% aqueous NH_4Ac and pH values ranging from 3 to 6.5 were tested. Under the conditions tested, all the analytes eluted within 30 min. When analysing extracts from buckwheat, signal interference was observed. A set of conditions was found under which signal suppression did not interfere with the chromatographic separation: [A] acetonitrile, [B] 0.010 M NH_4Ac , pH 4; flow rate 0.2 mL min^{-1} , gradient 15%-50% [B] over 16 min and isocratic 50% [B] for 10 min.

Sample preparation

To analyse D-fagomine in natural plant sources, we first studied its extraction from milled buckwheat groats and subsequent purification. Based on the information available concerning the solubility of D-fagomine [17] and our own tests, water/methanol (7:3) (120 mL per gram of buckwheat) was selected as the extraction solvent. Then, to purify the analyte, several SPE cartridges and conditions were tested: SCX (strong cation exchange), weak cation exchange, reversed phase and weak cation exchange–mixed mode. Only SCX cartridges were able to retain the added D-fagomine completely. To elute the D-fagomine, 2 M NH_3 solutions in water or methanol were assayed and only the aqueous solution was able to elute the D-fagomine. The processed samples were analysed by HILIC/ESI-Q-MS under the conditions described above. The maximum SPE loads were those in the aliquots from 100 mg of buckwheat groats.

Cation exchange HPLC/ESI-Q-MS for the separation of D-fagomine diastereomers

When analysing D-fagomine in buckwheat groats by HILIC/ESI-Q-MS, an extra signal at m/z 148.2 was observed which corresponds to a diastereomer that could only be partially separated from D-fagomine in the TSK-Gel Amide 80 column. The baseline separation of the

peaks required extremely long elution times resulting in MS signals that were too weak. A weak cation exchange setup was devised as an alternative to hydrophilic interaction. To optimize the separation conditions, we used a mixture of synthetic D-fagomine, 3-*epi*-fagomine and 3,4-di-*epi*-fagomine, since these isomers have been detected in other natural sources [15-17]. Using a TSK-Gel CM2SW column coupled to the ESI-Q-MS system, separation was achieved with aqueous NH₄Ac and a water miscible solvent (acetonitrile, methanol). To obtain baseline separation, mobile phases with different solvent composition (0 - 50% acetonitrile or methanol), ionic strength (10 - 60 mM) and pH (7.5 - 8.5) of the aqueous component, at different flow rates (0.7 - 0.9 mL min⁻¹) were tested. The best separation conditions were 50 mM aqueous NH₄Ac pH 8.5/methanol under isocratic conditions (4:1) at 0.8 mL min⁻¹.

The new method was applied to the analysis of buckwheat groats and mulberry leaves to detect the presence of D-fagomine and its naturally occurring diastereomers (Fig. 2). The extracts gave a signal at m/z 148.2 (t_R 14 min) with an elution time identical to that of a synthetic sample of D-fagomine obtained stereoselectively by the enzymatic method mentioned above (see Electronic Supplementary Material Figs. S3 and S4). In the case of buckwheat, the extra peak at m/z 148.2 (t_R 24 min) (Fig. 2a) was found to co-elute with synthetic 3,4-di-*epi*-fagomine. To confirm the identity of this isomer, which has never been reported in buckwheat before, a buckwheat groat extract was prepared and the compound was purified and analysed as described in the Materials and Methods section. The NMR results were compatible with 3,4-di-*epi*-fagomine or its enantiomer (5-*epi*-fagomine) (see Electronic Supplementary Material Figs. S1 and S2) and coincide with those described by Kato *et al.*, who assigned the signals to 3,4-di-*epi*-fagomine by NOE NMR [15]. In mulberry leaves, a different signal at m/z 148.2 (t_R 22 min) was observed (Fig. 2b). By comparison with a synthetic sample, this compound was identified as 3-*epi*-fagomine, which has previously been

1 reported in mulberry leaves [17]. A diastereomer (t_R 10 min) of DMDP (t_R 8 min) with the
2 exact mass confirmed by HPLC/ESI-HR-TOF-MS was also detected (Fig. 2c). Therefore, the
3 method proposed here may also be used to ascertain the diastereomeric purity of DMDP
4 standards. The molecular masses of D-fagomine and its diastereomers from buckwheat groats
5 and mulberry leaves were confirmed by HPLC/ESI-HR-TOF-MS.

6 The method was also applied to the separation of D-fagomine from its diastereomers and
7 related compounds from *Castanospermum australe* leaves and seeds. We identified 3-*epi*-
8 fagomine (t_R 22 min), 3,4-di-*epi*-fagomine (t_R 24 min) and one of the deoxy forms of D-
9 fagomine (m/z 132.2; t_R 20 min) (see Electronic Supplementary Material Fig. S5b,c). The two
10 isomers detected may correspond to the unidentified diastereomers reported previously [16].
11 Our results confirm the presence of D-fagomine and related compounds in *Castanospermum*
12 *australe* seeds and to our knowledge this is the first time these compounds have been reported
13 in *Castanospermum australe* leaves.

14 The new procedure presents several advantages over existing methods. As shown in Fig. 2, it
15 effectively separates D-fagomine from 3,4-di-*epi*-fagomine and 3-*epi*-fagomine in a single
16 run. Other analytical procedures (GC-MS, HILIC-MS) do not discriminate between D-
17 fagomine and its diastereomers [14,20] and may lead to overestimation of the amount of D-
18 fagomine in the matrices. Furthermore, the use of GC has the disadvantage of multiple peak
19 interference from the reducing carbohydrates in the matrices [32]. Under our cation exchange
20 HPLC conditions, monosaccharides and disaccharides (glucose, fructose, sucrose and lactose)
21 co-eluted shortly after injection (t_R 4 min) and did not interfere with the iminocyclitols.

22 23 Signal suppression effect and its influence on quantification

24

To evaluate the effect of possible interfering compounds in buckwheat groat samples, we studied the signal suppression effect of matrix components using D-fagomine solutions in a matrix-matched solvent. The equations of the signal/concentration curves obtained are included in Table 1. The matrix components caused D-fagomine signal suppression as evidenced by the smaller gradient obtained with the matrix-matched solutions. The use of DMDP as an internal standard allowed correction of the matrix effect, as its signal suffered nearly the same suppression. This demonstrates the need to correct measurements with an internal standard when analysing samples from complex matrices using ESI devices, as noted by other authors [33]. The signal suppression effect was also corrected in mulberry leaf samples by spiking with DMDP. The new method could then be used for the quantification of D-fagomine and its diastereomers in buckwheat and mulberry.

The signal suppression effect could not be avoided in the analysis of samples from *Castanospermum australe* leaves and seeds. Castanospermines (m/z 190.2) gave very intense signals at t_R 7, 10 and 15 min. These suppressed the D-fagomine and DMDP signals differently. Therefore, DMDP could not be used as an internal standard. To illustrate this suppression effect, selected ion monitoring (SIM) chromatograms (m/z 190.2, 132.2, 148.2, 164.2) from a DMDP spiked sample and standards are included as Electronic Supplementary Material Fig. S5. Other iminocyclitols, such as DGNJ (deoxygalactonojirimycin) (t_R 9 min) or DAB (1,4-dideoxy-1,4-imino-d-arabinitol) (t_R 21 min), may be considered as alternative internal standards for this matrix.

Method validation

The new method was validated for the determination of D-fagomine in buckwheat and mulberry. Linearity was studied for water solutions spiked with a fixed amount of DMDP to

correct for matrix effects (see Electronic Supplementary Material, Fig. S6). The assay response (area of the D-fagomine peak divided by the area of the DMDP peak) to D-fagomine concentration was linear between 4 and 40 mg kg⁻¹. Within-day precision and recovery were studied using samples of milled buckwheat groats, spiked with 10, 20 and 40 mg D-fagomine kg⁻¹. The percentage recovery at the three concentrations assayed (95%) was homogeneous (RSD = 8%), which demonstrates the accuracy and precision of the analytical procedure. The results are corrected for analyte loss during sample preparation through the use of the internal standard (DMDP). The limit of quantification (LOQ) was 1.5 mg kg⁻¹.

The results of the validation study show that the new method met all the requirements for a bioanalytical process [31]. The response was linear in the working range; recovery and precision fell within the recommended range; and the LOQ was much lower than the D-fagomine concentrations found in all the samples analysed except for buckwheat leaves. The method was successfully applied to the determination of D-fagomine and its diastereomers in different parts of the buckwheat plant (leaves, bran, groats and flour) from different producers and also in mulberry leaves.

Determination of D-fagomine in buckwheat and mulberry leaves

D-Fagomine and its diastereomer 3,4-di-*epi*-fagomine were determined in buckwheat groats, bran and leaves, as well as in buckwheat flour that is ready to use for the elaboration of foodstuffs (Table 2). Diastereomer content is expressed as D-fagomine equivalents.

Fagopyrum tataricum seeds contained less D-fagomine than *Fagopyrum sculentum*. 3-*epi*-Fagomine was not detected in any of the buckwheat samples. Deoxyfagomine (*m/z* 132.2), which has been detected in other sources of D-fagomine such as *Castanospermum australe* seeds [16], was not found in any of the samples analysed. The method was also applied to the

determination of D-fagomine and its diastereomers in mulberry leaves, where D-fagomine is not the major iminocyclitol. Apart from D-fagomine (m/z 148.2; t_R 14 min, 25 - 103 mg kg⁻¹) we identified and quantified 3-*epi*-fagomine (m/z 148.2; t_R 22 min, < LOQ - 12 mg kg⁻¹). The amount of D-fagomine and 3-*epi*-fagomine roughly coincides with the amounts reported by other authors : 50 – 660 mg kg⁻¹ [14] and 185 mg kg⁻¹ [17] in the case of D-fagomine and 21 mg kg⁻¹ in the case of 3-*epi*-fagomine [17].

In conclusion, we have devised a new cation exchange HPLC/ESI-Q-MS method to determine D-fagomine in buckwheat groats and other parts of the buckwheat plant. The new method has also been applied to mulberry leaves. It allows the separation of D-fagomine from 3-*epi*-fagomine and/or 3,4-di-*epi*-fagomine in a single run. We report the presence of 3,4-di-*epi*-fagomine in buckwheat for the first time. As the co-elution of D-fagomine and 3,4-di-*epi*-fagomine may lead to overestimation of the amount of D-fagomine, the new conditions will permit the accurate determination of this new functional component in plant samples and foodstuffs.

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Figure legends

Fig. 1 Chemical structures of: a, D-fagomine; b, 3-*epi*-fagomine; and c, 3,4-di-*epi*-fagomine.

Fig. 2 Cation exchange HPLC/ESI-Q-MS selected ion monitoring (SIM) chromatograms of samples from: a, buckwheat groats at m/z 148.2; b, mulberry leaves at m/z 148.2; and c, DMDP standard at m/z 164.2.

Figure 1

Figure 2

Table 1 Signal suppression effect in matrix-matched solutions and enhancement by spiking with DMDP

	D-fagomine signal vs D-fagomine concentration	D-fagomine/DMDP signal vs D-fagomine concentration
Aqueous solutions	$y = 43x - 33$	$y = 0.48x - 0.16$
Matrix-matched solutions	$y = 19x + 13$	$y = 0.45x + 0.060$
SSE	44%	95%

y , relative abundance; x , concentration in mg L^{-1} . SSE, signal suppression enhancement.

1 **Table 2** D-Fagomine and 3,4-di-*epi*-fagomine content in buckwheat.

	D-fagomine (mg kg ⁻¹)	3,4-di- <i>epi</i> -fagomine (mg kg ⁻¹)
Buckwheat (<i>F. sculentum</i>) groats (brand A)	44 ± 4	43 ± 3
Buckwheat (<i>F. sculentum</i>) groats (brand B, batch 1)	37 ± 2	31 ± 4
Buckwheat (<i>F. sculentum</i>) groats (brand B, batch 2)	22 ± 2	7.3 ± 0.3
Buckwheat (<i>F. sculentum</i>) groats (brand B, batch 3)	29 ± 1	24 ± 3
Buckwheat (<i>F. tataricum</i>) groats (brand C)	6.7 ± 0.3	1.0 ± 0.1
Buckwheat (<i>F. sculentum</i>) leaves (brand A)	< 1.5 (LOQ)	< 1.5 (LOQ)
Buckwheat (<i>F. sculentum</i>) bran (brand A)	19 ± 1	16 ± 2
Buckwheat (<i>F. sculentum</i>) bran (brand C)	27 ± 2	11 ± 0.7
Buckwheat (<i>F. sculentum</i>) flour (brand A)	41 ± 2	35 ± 4
Buckwheat (<i>F. sculentum</i>) flour (brand D)	29 ± 1	6.4 ± 0.3

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3 Values are means ± standard deviations, *n* = 3

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