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Determination of isoflavone glucoside malonates in *Trifolium pratense* L. (red clover) extracts: quantification and stability studies

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Abstract

Isoflavones, their glucosides and their glucoside malonates were determined in red clover leaf extracts using reversed-phase LC coupled to atmospheric pressure chemical ionisation mass spectrometry (APCI-MS), UV and fluorescence detectors and the stability of the malonates was investigated. Extracts can be stored at least 1–2 weeks at −20°C without loss of malonates. In LC-separated fractions the malonates are most stable when stored at low temperature after evaporation to dryness. The concentrations of eight major isoflavones ranged from 0.04 to 5 mg/g leaves.

Keywords: *Trifolium pratense* L.; Red clover; Stability; Glucoside malonates; Isoflavones

1. Introduction

Flavonoids are one of the most characteristic classes of compounds in higher plants. Many flavonoids are present as flower pigments in most angiosperm families. However, their occurrence is not restricted to the flowers but includes all parts of the plant. From a chemical point of view they comprise a wide group of structurally related compounds with a chromane skeleton provided with a phenyl substituent at the C_2 (flavones) or C_3 (isoflavones) position; the latter subclass is depicted in Fig. 1. Flavonoids are usually found in plants as glycosides, i.e., provided with sugar substituents such as galactose, rhamnose or glucose, or glycoside malonates. The malonates are of biological interest because the plant can use this conjugated form to store the less soluble flavonoid aglycons. Upon microbial infection, the aglycons are generated from these precursors by hydrolysis of the stored form [1]. Isoflavonoid phytoalexins play an important role in fungal infection, but isoflavonoids are also present in legumes, where they accumulate in the vacuoles either in their glycoside or their 6″-*O*-malonylated form [2]. Flavonoids may offer several benefits to human health, including antioxidant activity, metal chelation [3,4] and anticarcinogenic, antiallergic and antiviral effects [5]. They have also been shown to stimulate the immune system and to prevent nitration of tyrosine [6].

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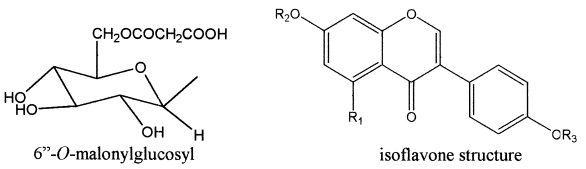

Name		R_1	R_2	R_3	MW
Daidzein	D	H	H	H	254
Daidzin (1)	DG	H	7-*O*-β-D-glc	H	416
Genistein	G	OH	H	H	270
Genistin (2)	GG	OH	7-*O*-β-D-glc	H	432
Formononetin (7)	F	H	H	CH_3	268
Ononin (3)	FG	H	7-*O*-β-D-glc	CH_3	430
Formononetin-7-*O*-β-D-glucoside-6”-*O*-malonate (4)	FGM	H	7-*O*-β-D-glc-6”-*O*-mal	CH_3	516
Biochanin A (8)	B	OH	H	CH_3	284
Sissotrin (5)	BG	OH	glc	CH_3	446
Biochanin A -7-*O*-β-D-glucoside-6”-*O*-malonate (6)	BGM	OH	7-*O*-β-D-glc-6”-*O*-mal	CH_3	532

Fig. 1. The structures of the 10 isoflavones studied and their abbreviations used in this paper; glc, glucoside; mal, malonate. The numbers in parentheses refer to the peaks in the chromatograms of Fig. 2.

Red clover (*Trifolium pratense* L.), also known as meadow, creeping or cow clover, is an important forage plant which grows in many parts of the world. It is an important feeding material for sheep and cattle and a health product for humans as medicinal herb for the treatment of eczema and psoriasis [7,8]. Many flavonoids, as well as glycoside and glycoside malonate derivatives of flavonoids have been identified in red clover and other species using reversed-phase liquid chromatography (RPLC) coupled with mass spetrometry [7,9,10]. Most workers have identified the same major flavonoids. However, in most studies no attention is paid to quantification and consequently, sample pretreatment, which is usually done by means of hydrolysis, is mentioned only briefly. Since it is our goal to study flavonoid profiles in wetland plants to use these as indicators for stress in wetland ecosystems, quantitative aspects of flavonoid analysis are very important. Therefore, in this work the stability and hydrolysis of red clover leaf extracts is investigated. For two selected major constituents of the extracts, the isoflavone glucoside malonates FGM and BGM (cf. Fig. 1), stability and degradation pathways were investigated using RPLC–UV, with the optimization of sample handling conditions as main objective. Subsequently, under optimised conditions, 10 major isoflavones were determined (and their presence confirmed) by using RPLC–UV with atmospheric pressure chemical ionisation (APCI)-MS and fluorescence detection (FL) for identification and additional confirmation.

2. Experimental

2.1. Materials

Daidzin, daidzein, genistin, genistein, formononetin and ononin were purchased from Roth (Karlsruhe, Germany), biochanin A and sissotrin from Indofine (Somerville, NJ, USA). Methanol, formic acid and HCl were from J.T. Baker (Deventer, The Netherlands), 4-hydroxy-1-naphthalenesulfonic acid and ammonium formate from Aldrich (Steinheim, Germany), tris(hydroxymethyl)-aminomethane (Tris) from Merck (Darmstadt, Germany). Ultrapure water was prepared with a Millipore–Academic system (Etten-Leur, The Netherlands). Extracts were made using fresh leaves of *T. pratense* from plants collected in a field (Ouddorp, The Netherlands) and grown in a greenhouse.

2.2. Extraction and isolation procedures

The procedure for the extraction of flavonoids from red clover was adapted from Toebes et al. [11]. A 500-mg amount of fresh leaves was extracted in an ultrasonication bath for 60 min at room temperature with 10 ml of methanol–water (9:1, v/v) and 3 ml of aqueous 350 m*M* Tris buffer. The extracts were filtered over a 0.45-μm filter before pretreatment and/or injection.

Fractions containing the two most dominant peaks, FGM and BGM, were collected after LC separation by collecting 1 ml of column eluent, at their peak maximum on the UV detector. The samples were hydrolysed by heating the filtered extracts in a sealed vial up to 4 h at 83°C in the presence or absence of concentrated HCl.

2.3. RPLC–UV and –FL

LC was performed on a two-pump system equipped with a dynamic mixer and a manometric module (Gilson Medical Electronics, Villiers-le-Bel, France). UV and fluorescence data were obtained with a Hewlett-Packard Series 1050 UV detector (set

Table 1
LC gradient used for isoflavone analysis

Time (min)	% Methanol	% Buffer
0	30	70
5–10	45	55
15	50	50
25	55	45
30	60	40
35–40	99	1
42	30	70

at 265 nm) and an Applied Biosystems (Foster City, CA, USA) Model 980 programmable fluorescence detector (λ_{ex}=250 nm, emission filter at 418 nm). A 250×4.6 mm I.D. 5 μm Zorbax SB-C_{18} column was used. The eluent consisted of a mixture of methanol–aqueous 10 m*M* ammonium formate buffer, pH 4.0. All solvents were filtered and degassed with helium before use. The LC gradient is shown in Table 1. The flow was 1.0 ml/min and the injection volume, 10 μl.

2.4. RPLC–MS

Analyses were performed on a Shimadzu (Princeton, NJ, USA) LC system, consisting of two LC-10A LC pumps, a DGU-14A degasser, an SIL-10AD auto-injector, an SCL-10A system controller unit and an SPD-10A UV detector (set at 265 and 290 nm), coupled to a ThermoQuest Finnigan (San Jose, CA, USA) LCQ deca ion trap mass spectrometer. The same gradient as in LC–UV was applied. APCI-MS spectra were acquired in the negative ion mode in the *m*/*z* range of 150–650. Only in an exceptional case, the positive ion mode (also in the *m*/*z* range 150–650) was shown to be more favourable (see Fig. 6). The source and probe temperatures were maintained at 150 and 450°C, respectively, and the activation energy at 30%. The LC flow was directed into the mass spectrometer without stream splitting.

3. Results and discussion

The two main isoflavones in red clover are FGM and BGM which have been characterized in several MS-based studies as isoflavone glucoside malonates [2,7]. They are subject to decomposition according to:

Formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate (FGM) → ononin (FG) → formononetin (F) (1)

and

Biochanin A-7-*O*-β-D-glucoside-6″-*O*-malonate (BGM) → sissotrin (BG) → biochanin A (B) (2)

Below, first of all LC conditions are presented under which the above decomposition is expected to be largely absent. Next, the hydrolysis of FGM and BGM is studied under either mild or harsh conditions and their stability is investigated. Finally, quantification of the analytes of Eqs. (1) and (2) and, also, the isoflavone glucosides, daidzin and genistin, and their respective aglycons, daidzein and genistein, was performed by RPLC–UV with identification and additional confirmation by RPLC–APCI-MS and fluorescence.

3.1. LC separation

Since the present study focuses on identification as well as quantification, we used gradient LC conditions, which are compatible with UV as well as MS detection. The somewhat complicated gradient of Table 1 was found to be fully satisfactory in this respect. Its optimisation will be discussed in detail in a parallel paper [12]. Here it should be noted that contrary to Lin et al. [7] who used an acidic eluent (water and acetonitrile containing 0.25% acetic acid), we preferred aqueous ammonium formate at pH 4.0. In our experience such conditions are quite suitable for MS detection by means of, e.g., APCI or electrospray ionsiation (ESI) in the negative mode.

The LC–UV, LC–MS and LC–FL chromatograms of a red clover extract – independently recorded, using different experimental set-ups – are shown in Fig. 2. The LC–UV chromatogram was recorded at 265 nm, a good compromise wavelength for the isoflavones of Fig. 1, which all have absorption maxima in the 255–270 nm range. For FGM and BGM no standards were available but, most probably, their absorption characteristics hardly differ from those of FG and BG, respectively, since the

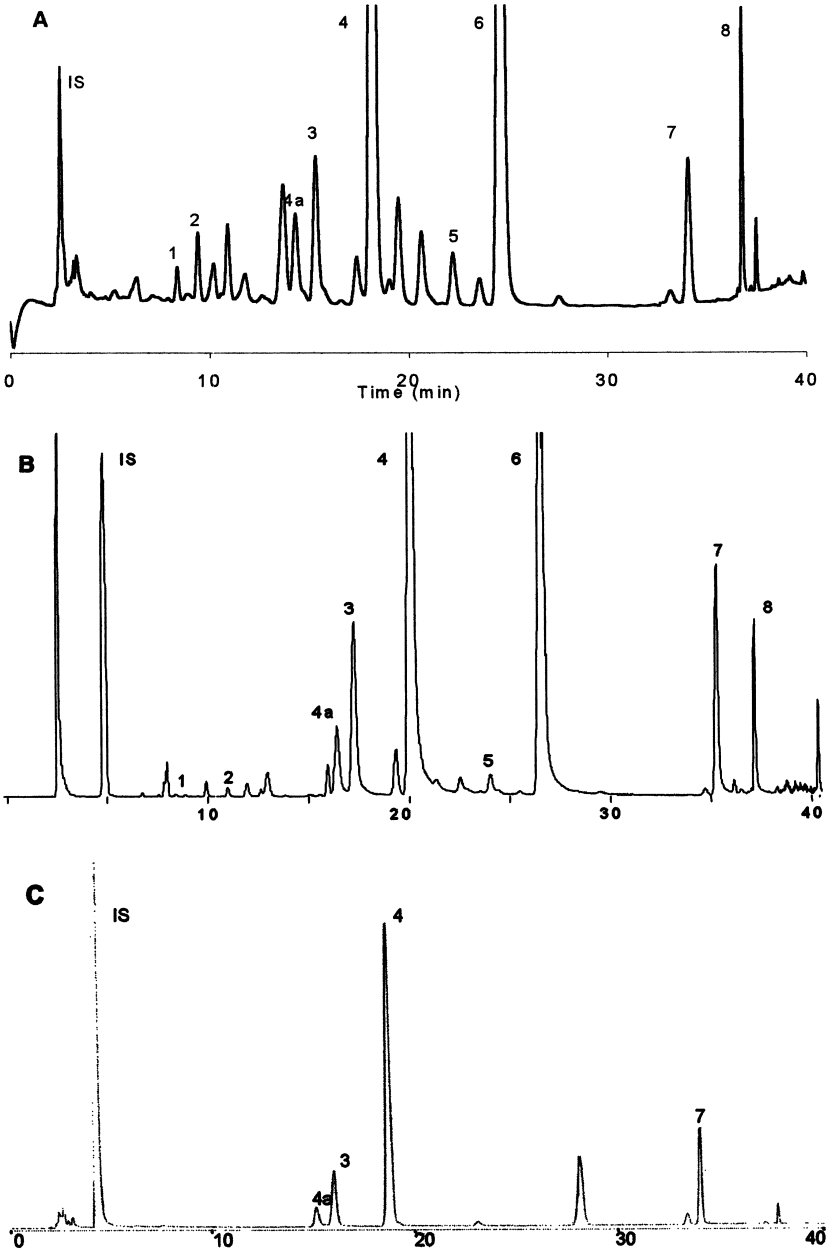

Fig. 2. Independently recorded (A) LC–UV_{265}, (B) LC–APCI-MS (negative mode; full scan) and (C) LC–FL chromatograms of *T. pratense* L. extract. Peak 4a is an FGM isomer, see text. Peak numbers refer to Fig. 1. The small peaks 1 and 2 in B were recognized in SIM. Vertical axes are in relative units.

chromophoric skeletons will hardly be influenced by the loss of the malonate group.

The LC–MS chromatogram serves to check whether in Fig. 2A indeed an isoflavone profile is recorded with other sample constituents playing a minor role. Comparison of the two chromatograms shows that the profiles are similar, as is illustrated by the peak numbers referring to the isoflavones listed

in Fig. 1. With LC–MS, 24 peaks were recorded (not all visible in Fig. 2B) using selected ion monitoring (SIM). More importantly, they could all be attributed to flavones, as will be discussed in more detail in a forthcoming paper [12]; these results generally agree with those of Lin et al. [7]. The intense peaks in the LC–UV chromatogram eluting at 18.2 and 24.6 min, which also dominate the LC–MS chromatogram, can be attributed to FGM and BGM, respectively, the compounds of main interest in the present study. The LC–FL chromatogram (Fig. 2C) provides additional selectivity. As is to be expected, most isoflavones do not show native fluorescence. Only five prominent peaks can be seen in the fluorescence chromatogram. Three of them can be attributed to FG (3), FGM (4) and F (7). Peak 4a is – also in view of its mass spectrum – attributed to an isomer of FGM. The peak at 28 min probably is pratensein, but this could not be confirmed because no pratensein standard was available.

3.2. Hydrolysis of FGM and BGM

In the literature quantification of flavonoid glycosides and glycoside malonates has not received much attention, although there are some exceptions [2,10,13]. In these studies quantification is simply based on the formation of aglycons after hydrolysis, and speciation is not considered. In this paper we use FGM and BGM to study the hydrolysis process (cf. Eqs. (1) and (2)) in some detail. In order to do rapid screening of many samples, the hydrolysis procedure of choice should be efficient and not too time-consuming. LC fractions containing only FGM and BGM were collected to prevent interfering reactions from other sample constituents as much as possible. These fractions were hydrolysed by heating without and with acidification (cf. Ref. [7]).

3.2.1. Heating without acidification

FGM and BGM fractions prepared as described in Section 2.2 were heated at 83°C. Injections of the (partly) hydrolysed fractions were made every 30 min over a period of 2 h. Changes in the concentrations of BGM, FGM, ononin (FG), sissotrin (BG), formononetin (F) and biochanin A (B) were monitored on the same LC system as used above. Prior to injection 20 μl of a 1000 μg/ml 4-hydroxy-1-naphthalene sulfonic acid solution was added to 200 μl of each fraction as an internal standard (I.S.). Typical results are shown in Fig. 3. Responses are expressed as the ratio of the peak areas of the analytes and the I.S.. The data show that both FGM and BGM completely disappear within 2 h. The corresponding glucosides, FG and BG, show a simultaneous concentration increase. Since the UV signals, i.e., the concentrations of the aglycons F and B remain essentially constant at a very low level – there are always some aglycons present in the sample that are formed during extraction – one can conclude that, in the absence of acid, quantitative conversion of the parent compounds into the relatively stable glucosides FG and BG can easily be achieved. Heating for more than 2 h is not advisable because the data of Fig. 3A and C suggest that the glucosides then begin to decompose (which may well considerably influence results obtained after 16 h heating such as reported in Ref. [7]).

3.2.2. Heating with acidification

Similar experiments were performed in which heating at 83°C was carried out after the addition of 40 μl of concentrated HCl to 200 μl of sample. LC analysis was done in the same way as above. Quite different results were obtained. Fig. 3 shows that, in the presence of HCl, degradation of BGM and FGM is more rapid, and is taken one step further. The parent compounds are lost within 60–90 min, but the glucosides which are formed, are subsequently rapidly converted to the aglycons F and B. This conversion is complete in about 2 h, but the aglycons themselves also show degradation, as is shown by the analyses of the 4-h-treated samples. Obviously, for quantification purposes hydrolysis in the absence of acid is to be preferred.

3.3. Stability of FGM and BGM in extracts of leaves

For longer-term studies, it is important to know whether or not the intact malonates, FGM and BGM, are stable for prolonged periods of time, either in the extracts themselves or in the fractions collected after LC separation.

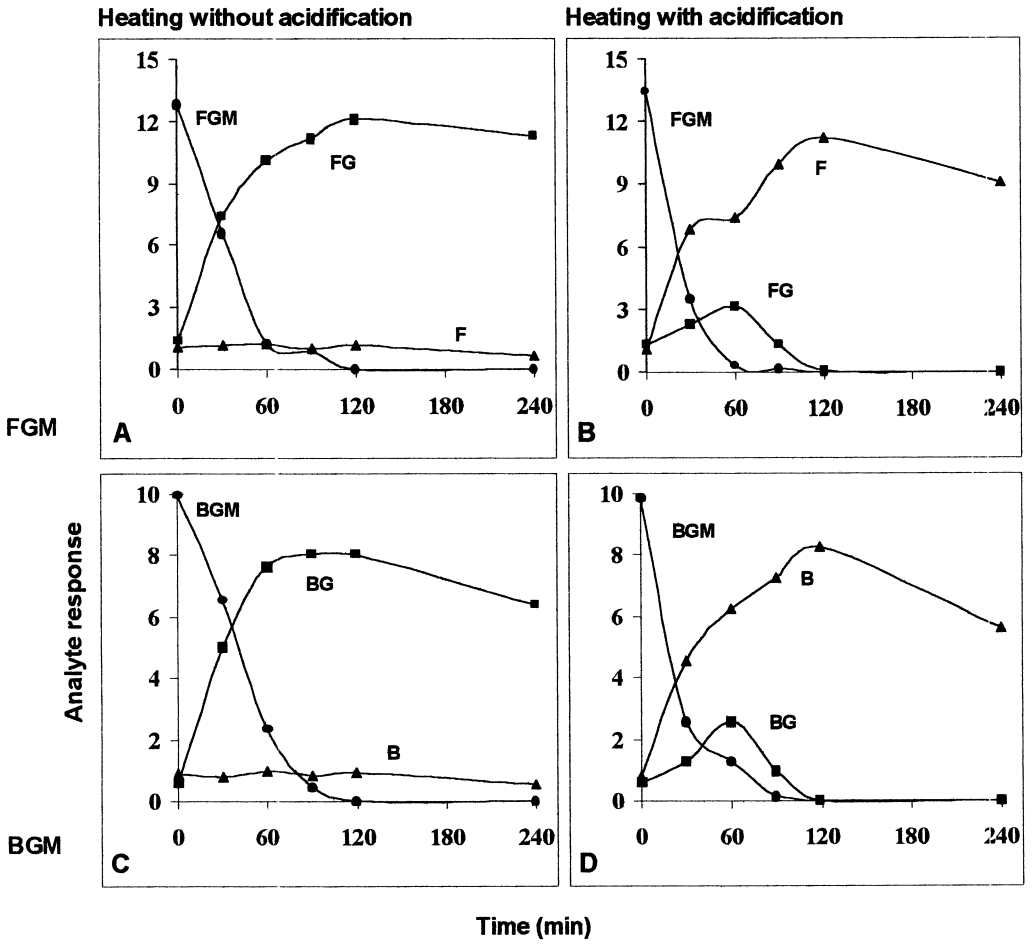

Fig. 3. Effect of hydrolysis on the degradation of FGM (A and B) and BGM (C and D). (A and C) by heating at 83°C and (B and D) by heating at 83°C in the presence of HCl. For LC conditions, see text.

3.3.1. Stability in extracts

A red clover extract in methanol containing 100 μg/ml of I.S. was divided into three portions. One of these was stored in the freezer at −20°C and another one at room temperature. The third portion was stored in the freezer after evaporation to dryness under nitrogen. Changes in the composition of the three samples were studied over a period of 2 weeks and monitored by means of LC–UV. The results displayed in Fig. 4 show that, in the solution stored in the freezer, the malonates are stable for at least 1–2 weeks. However, if the sample is kept at room temperature and, somewhat surprisingly, also if it is stored dry at −20°C, degradation starts to occur almost immediately. Under these conditions, the malonate group is lost, but the glucosides remain intact. The decrease in the malonate concentrations in dried samples may be due to incomplete evaporation of the eluent, i.e., the continued presence of part of the buffer constituents.

In all further studies, we used solutions of extracts that had been stored at −20°C for a maximum period of 1 week.

3.3.2. Stability in LC-separated fractions

The LC fractions containing FGM and BGM were collected as described above. Aliquots of these fractions dissolved in the methanol–formate buffer used as LC eluent were stored in the freezer and injected at $t=0$, 4, 7 and 14 days. Other portions of

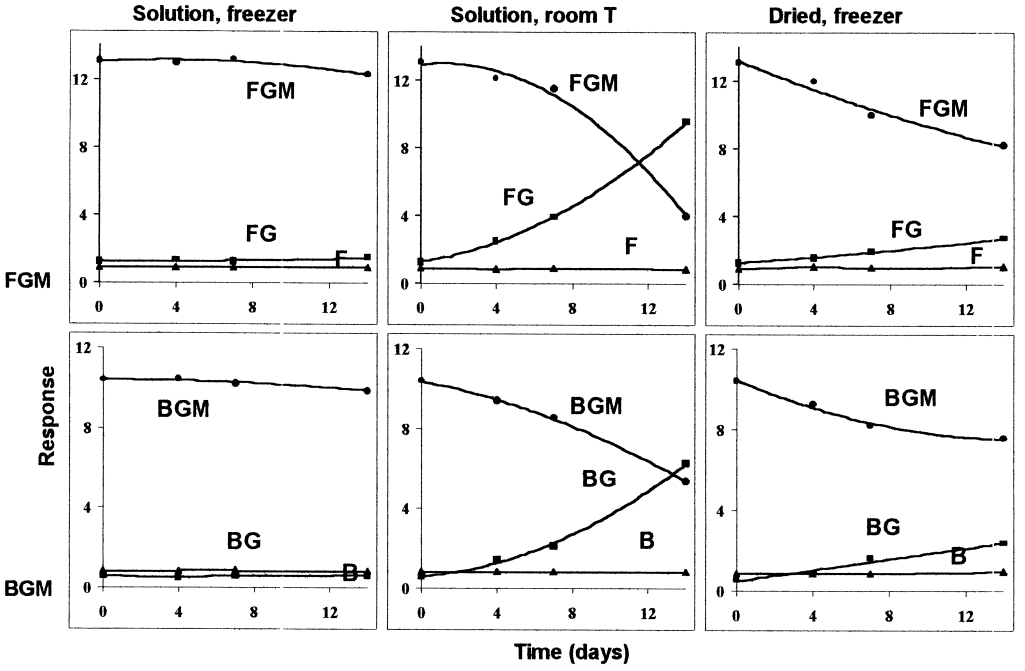

Fig. 4. Stability of FGM and BGM in a red clover extract under different storage conditions. For details and LC conditions see text.

the same fractions were evaporated to dryness under nitrogen at room temperature. One part was redissolved in methanol immediately after evaporation and was then stored in the freezer. Another portion was stored dry in the freezer and redissolved in methanol just prior to injection. It will be clear that the latter portion had to be taken from the freezer prior to analysis and thawed; next, after dissolving it in methanol and taking an appropriate volume for injection and LC analysis, the remaining solution had to be evaporated again. That is, there was more manipulation than with the other samples. The results of Fig. 5 show that the malonates are not stable when stored at −20°C in methanol–formate buffer: within 24 h they are completely decomposed. Stability is also poor for samples dissolved in methanol after evaporation of the eluent, but complete decomposition now takes several days. The best option is to store the fractionated malonates dry and redissolve them just prior to injection. However, even then the stability is not as good as observed for the original extracts stored at low temperature (cf. Fig. 4). Similar results were obtained when evaporation was carried out with helium instead of nitrogen.

In summary, extracts can be stored at least 1–2 weeks at −20°C without loss of malonates. In separated LC fractions the stability is invariably poorer, with the best results being obtained for samples stored dry at −20°C. Possibly, the malonate decomposition is caused by a less than complete removal of the eluent plus buffer during evaporation. This would explain why the results are somewhat better with the repeated dissolution and evaporation procedure.

3.4. Fluorescence

As noted above, 10 isoflavones were selected to characterize the clover leaf extracts (cf. Fig. 1). Unfortunately, they all have the same ‘‘chromophoric skeleton’’ and therefore quite similar UV absorption characteristics. In other words, UV detection provides hardly any selectivity. The situation is, however, completely different when fluorescence detection is used. In fact, the number of isoflavones that show native fluorescence is quite limited. Therefore LC–FL is potentially useful for qualitative studies.

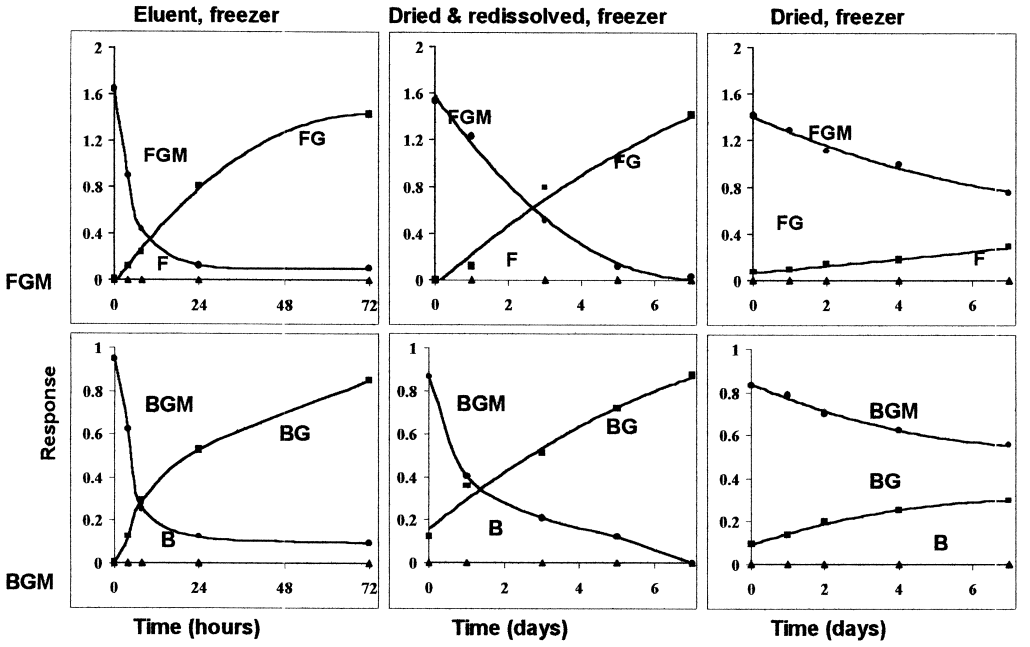

Fig. 5. Stability of FGM and BGM in LC-separated fractions under different storage conditions. For details and LC conditions see text.

It was found that, from among the ten isoflavones mentioned above, FGM, FG and F emit strong fluorescence, in contrast to BGM, BG and B which are non-fluorescent (cf. Fig. 2). Daidzin (DG) and daidzein (D) deserve special attention. Standard solutions of both DG and D displayed fluorescence. However, when the DG standard was analysed by LC–MS, a much smaller peak was detected with the same mass as DG, which co-elutes on the tail of the daidzin peak but has a different fragmentation pattern (Fig. 6). Further experiments showed that it is this isomer, which is present in the DG standard at very low concentration, that causes the relatively high fluorescence signal. In fact DG itself did not show any fluorescence at all. The excitation maximum of the isomer is at 240 nm, the same as for D. As far as we know this point did not yet receive any attention in the literature.

3.5. Quantification

Quantification of the 10 isoflavones was carried out using LC–UV. FGM and BGM, of which no standards are available, were quantified after hydrolysis to ononin and sissotrin, respectively. Calibration plots were constructed by injecting standard solutions containing between 0.10 and 125 μg/ml of the analytes (10 data points in triplicate). R values were between 0.9998 and 0.9980. Detection limits ($S/N=3$; $n=7$) are presented in Table 2; they were between 20 and 90 μg/ml. Repeatability data are also shown in Table 2: for standard solutions all RSD values of the peak areas ($n=7$) were below 10%.

For the clover extracts all analyses were carried out in triplicate. Table 2 summarises the results. For the sake of convenience, the concentrations are expressed in μM, since analyte conversions (after hydrolysis) are considered. As is to be expected for a glucoside, the concentration of daidzin remains essentially the same after the non-acid hydrolysis, and becomes zero after acid hydrolysis. Daidzein was not detected in the clover extract, but was formed as a result of 1 M acid hydrolysis due to loss of the glucose group of daidzin. The same behaviour is found for the glucoside/aglycon pair genistin/genistein. The conversion yield was about 80% for the genistin/genistein pair and about 30% for daidzin/daidzein. Probably, genistein and, much more so, daidzein undergo further decomposition. Further

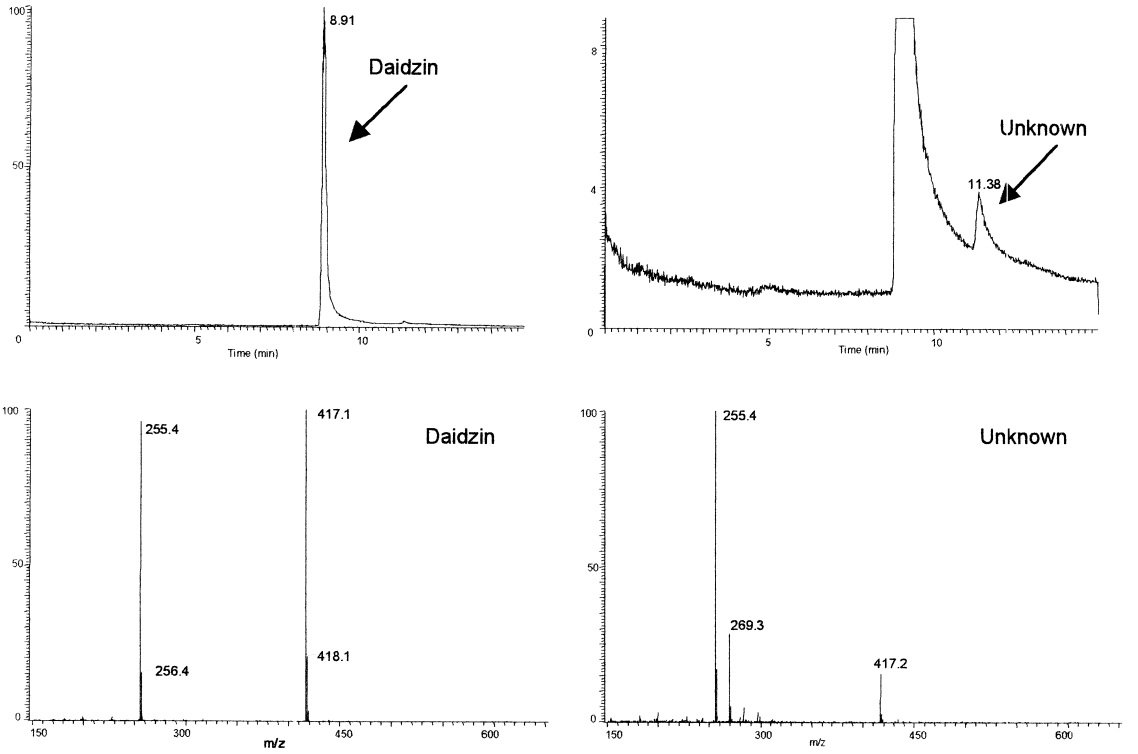

Fig. 6. LC–APCI-MS chromatogram (positive mode; full-scan) of daidzin standard (t_R 8.91 min) and co-eluting isomer (t_R 11.38 min) with corresponding mass spectra. For details see text.

study of this aspect was beyond the scope of the present project.

FGM and BGM were determined by subtracting the concentrations of FG and BG before non-acid hydrolysis from those found after this treatment. If heating is performed under acidic conditions, the glucosides are degraded further into their corresponding aglycons, a somewhat different situation as

Table 2
Quantification of isoflavones in clover leaves; the limit of detection (LOD) and RSD columns refer to standard solutions

Analyte	LOD (μg/ml)	RSD of peak area (%, $n=7$)	Concentration in initial extract (μM)	Concentration in extract after heating (μM)	Concentration in extract after heating + HCl (μM)	Concentration in leaves (μg/g)
Daidzin	35	5	1.8	1.9	0.0	42
Daidzein	70	5	0	0	0.6	0
Genistin	85	6	25	26	0	560
Genistein	90	7	0	0	21	0
FGM	–	–	186*	0	0	4900
Ononin	80	4	44	230	0	970
Formononetin	65	2	43	45	260	600
BGM	–	–	117*	0	0	3300
Sissotrin	50	3	23	140	0	540
Biochanin A	20	4	23	27	130	330

*Calculated values, see text.

was explained above in the context of Fig. 3B and D. With the FGM/FG/F series, the final formononetin concentration of 260 μ*M* closely matches the summed concentration of the three compounds in the initial extract (270 μ*M*). With the BGM/BG/B series there is some discrepancy, (130 vs. 163 μ*M*) which probably reflects some further degradation, also mentioned above.

4. Conclusions

Sample hydrolysis and storage conditions have been optimised to enable the quantification of isoflavones in clover leaves. Storage is best done at −20°C: for at least 1–2 weeks no significant loss of malonate esters is observed. Hydrolysis at elevated temperatures (83°C) should preferably be performed over a 2-h period without acidification. Under such conditions FGM and BGM are quantitatively converted into the corresponding glucosides while decomposition of the latter is still negligible. Hydrolysis at 83°C in the presence of acid provides a way to obtain additional information on the flavonoid distribution. In the present study, LC–APCI(−)-MS under conditions which are rather mild (pH 4.0) compared to those sometimes used in the literature proved a good means for analyte identification. Preliminary experiments using LC–FL indicate the selectivity of this technique in flavonoid studies; it is currently the subject of further investigations. For quantification LC–UV was used for 10 target compounds comprising aglycons, glucosides and glucoside malonates. For eight out of the 10 target analytes, the concentrations in the clover leaves were found to range from 0.042 μg/g to 4.9 mg/g which opens the possibility to use these compounds as indicators to characterise wetland ecosystem properties.

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References

[1] L.W. Sumner, N. Paiva, R.A. Dixon, P.W. Geno, J. Mass Spectrom. 31 (1996) 472.
[2] R. Edwards, S.A. Tiller, A.D. Parry, J. Plant Physiol. 150 (1997) 603.
[3] J.E. Brown, H. Khord, R.H. Hider, C.A. Rice-Evans, Biochem. J. 40 (1992) 1591.
[4] C.A. Rice-Evans, N.J. Miller, G. Paganga, Free Radic. Biol. Med. 20 (1996) 933.
[5] E. MiddeltonJr., C. Kandaswami, J.B. Harborne (Eds.), The Flavonoids: Advances in Research Since 1986, Chapman and Hall, Cambridge, 1994, p. 619, Chapter 15.
[6] C. Oldreive, K. Zhao, G. Paganga, B. Halliwell, C. Rice-Evans, Chem. Res. Toxicol. 11 (1998) 1574.
[7] L.Z. Lin, X.G. He, M. Lindenmaier, J. Yang, M. Cleary, S.X. Qiu, G.A. Cordell, J. Agric. Food Chem. 48 (2000) 354.
[8] B. Klejdus, D. Vitamvásová, V. Kubán, J. Chromatogr. A 839 (1999) 261.
[9] U. Justesen, J. Chromatogr. A 902 (2000) 369.
[10] W. Andlauer, M.J. Martena, P. Fürst, J. Chromatogr. A 849 (1999) 341.
[11] A.H.W. Toebes, P.L.M Koevoets, H. Lingeman, J.A.C. Verkleij, W.H.O. Ernst, manuscript in preparation.
[12] E. de Rijke, H. Zappey, F. Ariese, U.A.Th. Brinkman, C. Gooijer, manuscript in preparation.
[13] H. Chen, Y. Zuo, Y. Deng, J. Chromatogr. A 913 (2001) 387.