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Uptake of the pharmaceutical Fluoxetine Hydrochloride from growth medium by *Brassicaceae*

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ABSTRACT

Since the European Union banned disposal of sewage sludge (SS) at sea in 1998 the application rate of SS to land has risen significantly. Land application is thus possibly an important transport route for SS-associated organic chemicals, including pharmaceuticals, to soils and perhaps also to plants. The potential for the selective serotonin re-uptake inhibitor, Fluoxetine HCl, to undergo uptake into *Brassicaceae* tissues was therefore investigated in a tissue culture study under laboratory conditions for 12 weeks. From growth medium containing 280 ng Fluoxetine HCl mL^{-1}, translocation into *Brassica oleracea* var. *botrytis* (cauliflower) stems (5% mean uptake of applied burden; 0.49 μg g wet $weight^{-1}$) and leaves (3% mean uptake; 0.26 μg g wet $weight^{-1}$) was confirmed, but no evidence of uptake into the curd was found; other possible explanations of the observations are also discussed. Although the data for individual plants were highly variable, as was the recovery of spiked internal standard (deuterated Fluoxetine HCl), the results nonetheless suggest uptake of Fluoxetine may indeed be a potential transport route to plants. A similar study of uptake from soils rather than from an artificial medium should now be undertaken, with greater numbers of replicates and improved analytical methods. Such studies have already demonstrated uptake of some antibiotics from manured soils by a variety of plants including *Brassicaceae*, suggesting that the uptake mechanisms may be more general.

1. Introduction

During the last decade the occurrence of pharmaceuticals and personal care products (PPCPs) in the environment has been well documented (e.g. Halling-Sorensen et al., 1998; Heberer, 2002; Ternes, 1998; Daughton and Ternes, 1999). A range of studies has shown that some PPCPs are neither completely removed by sewage treatment works (STW) processes, nor completely degraded in the environment; hence the environmental occurrence of PPCPs is perhaps of little surprise (Carballa et al., 2004; Golet et al., 2002; Heberer et al., 2001).

Since the European Union (EU) banned disposal of sewage sludge (SS) at sea in 1998, the application rate of SS to land has risen significantly and is set to rise further. Fifty-two percent of SS was disposed of to land in the UK in 2000. Land application is thus possibly an important transport route for SS-associated organic chemicals such as PPCPs, into the environment (reviewed by Jjemba, 2002).

Thus far, most research has focussed on monitoring, biodegradation and sorption studies of PPCPs, but the possible uptake into plants from treated soils has also been highlighted (Migliore et al., 1995; reviewed by Jjemba, 2002) and a few recent, and some older, studies have appeared (e.g. Royse et al., 1975; Migliore et al., 1995, 1996, 1997; Boxall et al., 2006; Dolliver et al., 2007). These have been mainly concerned with the uptake of veterinary pharmaceuticals, such as antibiotics, from soils treated with animal manure (e.g. Kumar et al., 2005; Dolliver et al., 2007). A few of these PPCPs are also used as human medicines (Boxall et al., 2006) but data for the uptake by plants of PPCPs used to treat humans are still rather sparse. It has been recommended that more intensive experimental work to explore the mechanisms of uptake of a range of medicines by plants should be undertaken and that the use of hydroponics and sampling of sap rather than whole plants should be performed (Boxall et al., 2006).

We therefore selected for study, the pharmaceutical Fluoxetine HCl (sometimes known by the one of the tradenames, Prozac®). Fluoxetine HCl is widely-prescribed for human use and has featured on the top 200 most prescribed drugs list for the USA since 1995, in the UK top 100 prescribed pharmaceuticals by mass, with 2.83 tonnes of Fluoxetine HCl dispensed in 2000, and it has been predicted that over 34 million people have taken the drug in over 100 countries (Fong, 2001). Fluoxetine HCl might be expected to undergo plant uptake based on a pH-dependent octanol–water

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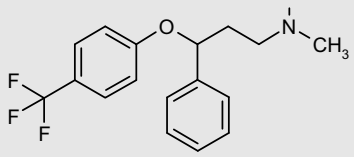
Table 1
Structure and some relevant physico-chemical properties of Fluoxetine (HCl)

Compound	Molecular weight	Log K_{ow}	Log K_{oc}	BCF	Aqueous solubility (mg L^{-1})	PEC/PNEC	Environmental reports
Fluoxetine (hydrochloride) 	309 (345)	1.25–4.3 (pH 2–11)[a] 4.05[b]	0.64–3.70 (pH 2–11)[a]	1;2.00;1071.52 (pH 2;7;11)[a]	60.3[b]	14.19[c] EU PEC[d] 0.22 μg L^{-1}	STW effluents[e,f] 0.038–0.099 μg L^{-1}, <LOQ-1.3 ng L^{-1}
						US EIC[d] 0.23 μg L^{-1}	DWTP[g] solids 49.5 μg kg^{-1}

Abbreviations: K_{ow}, octanol–water partition coefficient; K_{oc}, organic carbon-normalised partition coefficient; BCF, bioconcentration factor; PEC/PNEC, predicted environmental concentration (EU)/predicted no-effect concentration (EU); STW, sewage treatment works; LOQ, limit of quantitation; DWTP, drinking water treatment plant; and EIC, environmental introduction concentration.

[a] Brooks et al. (2003).
[b] Kinney et al. (2006).
[c] Webb (2001).
[d] Johnson et al. (2005).
[e] Metcalfe et al. (2003).
[f] Vasskog et al. (2006).
[g] Stackelburg et al. (2007).

partition coefficient (K_{ow}) of between 1 and 4 (Table 1; Brooks et al., 2003; Kinney et al., 2006) although K_{ow} is not always a good predictor of plant uptake (Boxall et al., 2006). In order to undergo plant uptake a PPCP must also be stable within the soil system for a sufficiently long period ($t_{1/2}$ > 14 days; O'Connor, 1996). Fluoxetine HCl has been shown to be resistant to biodegradation in soils for up to 270 days (Redshaw, 2008). The chemical structure and some relevant physical characteristics of Fluoxetine HCl are shown in Table 1.

Plants used to study the uptake of PPCPs to date have included corn (*Zea mays* L.), potato (*Solanum tuberosum* L.), lettuce (*Lactuca sativa* L.), and cabbage (*Brassica oleracea*; Kumar et al., 2005; Boxall et al., 2006; Dolliver et al., 2007). Cauliflower (*B. oleracea* var. *botrytis*) is a further important economic vegetable crop which might legally be grown on SS-amended soils. In 2005 more than 11 million tonnes of cauliflower was produced worldwide with more than 130,000 tonnes produced in the UK (DEFRA; http://www.statistics.defra.gov.uk; accessed 2007).

Given the recommendations made previously, in the present study we measured the uptake of Fluoxetine into different parts of cauliflower plants, grown in artificial media. Thus, Fluoxetine concentrations in roots, stems, leaves and curds of tissue cultures (T/C) of cauliflower after 12 weeks growth, were determined by high performance liquid chromatography–electrospray–mass spectrometry (HPLC–ESI–MS).

2. Results and discussion

2.1. Growth rates

Cauliflower tissue masses were recorded prior to tissue culture and at harvest so that growth masses (Table 2) could be determined and any potential phytotoxic effects upon growth rates identified. Results of statistical analyses using *F*-test and *t*-tests showed no significant differences in either the means or variance, between spiked and blank sample sets for the whole plant, or individual sub-samples, at the 95% confidence interval. The data did indicate a possible phytotoxic effect on the growth of the roots of Fluoxetine-treated plants (Table 2) but the low masses of the roots meant that errors in measurements and hence the standard deviations (Table 2) were too large for the differences to be statistically significant.

Thus there was no evidence of phytotoxic impacts upon the growth masses of cauliflower T/C exposed to Fluoxetine HCl. It is known that the phytotoxic effects of therapeutic agents differ for different PPCPs and crop species (reviewed by Jjemba, 2002; Boxall et al., 2006).

Table 2
Harvest and calculated growth data for Fluoxetine exposed and non-exposed cauliflower tissue cultures (n = 4 or 5)

Harvest mass (g)							
Sample	Root	Stem	Leaf	Curd	Total	Original T/C section mass (g)	Growth mass (g)
Exposed samples							
Mean	0.23	1.29	2.08	1.21	4.39	1.07	3.32
Standard deviation	0.34	0.48	1.59	0.45	2.62	0.11	2.57
% RSD	152	37	76	37	60	11	77
Blank samples							
Mean	0.81	1.19	1.79	1.07	4.69	1.07	3.61
Standard deviation	0.17	0.52	0.72	0.29	1.65	0.20	1.60
% RSD	22	44	40	27	35	18	44

2.2. Uptake and translocation

Extracts of cauliflower T/C were examined by HPLC–ESI–MS in positive ion mode using selective ion monitoring (SIM). Peaks in the extracted ion chromatograms that were positively identified as Fluoxetine by selective reaction monitoring (SRM) (m/z transition 310–147.9) and retention time (R_t) were integrated and these data used to obtain quantitative data on uptake (Table 3). Due to the known limitations of electrospray ionisation – ion trap mass spectrometers for the generation of quantitative data, calibration involving ratio calibration and matrix matching was performed. Ratio calibration using deuterated internal standards was found to be suitable for the production of quantitative data for the target compounds (Redshaw, 2008). Matrix-matched calibration was used to investigate ion suppression by the matrices resulting from extraction of plant material (specifically cauliflower tissues) on the response of Fluoxetine and d_5-Fluoxetine. Matrix matching did not produce a statistically significant advantage over the use of non-matched ratio calibration (ANOVA F-ratio = 0.0024, P-value = 1.00 > 0.05).

Table 3 and Fig. 1 summarise data for the uptake of Fluoxetine into cauliflower T/Cs. Positive identification of Fluoxetine in all media and stem samples was achieved and from two of the four leaf samples. No Fluoxetine was identified in any of the

Table 3
Fluoxetine uptake to cauliflower tissue cultures

	Positive identification	Range (%)[a]	Mean (%)	Standard deviation (of %)	Concentration (μg g^{-1} wet weight)	Concentration (μg mg^{-1} lipid)
Leaves	2 from 4	0–7	3	3.5	0.26	0.03
Curd	0 from 5	<LOD	<LOD	<LOD	<LOD	<LOD
Stem	5 from 5	3–8	5	2.4	0.49	0.29
Roots	0 from 4	<LOD	<LOD	<LOD	<LOD	<LOD
Media	5 from 5	5–50	23	20.3	n/a	n/a

n/a, not applicable and LOD, limit of detection.
[a] Percentage of total 9.8 μg Fluoxetine added to each growth pot.

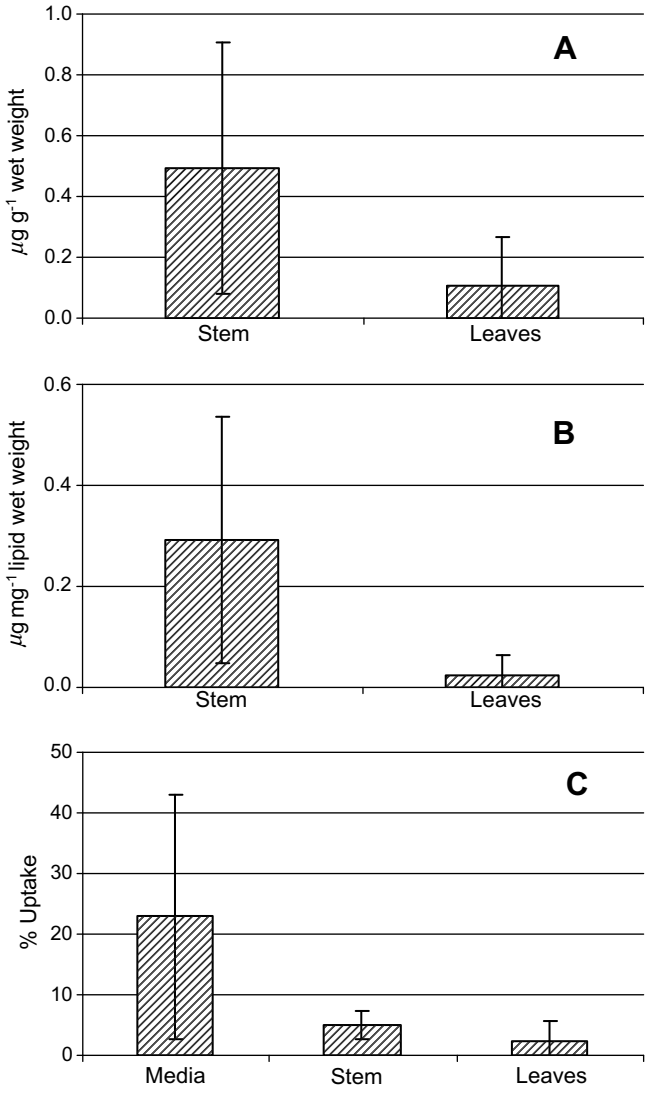


Fig. 1. Summary of Fluoxetine uptake to cauliflower tissue cultures and amounts in residual media. (A) Fluoxetine μg per gram of wet cauliflower tissue; (B) Fluoxetine μg per milligram of lipid of tissue; and (C) percentage uptake of initial Fluoxetine added (9.8 μg) and percentage residue in media. For media and stem samples, $n = 5$. For leaf samples, $n = 4$.

non-exposed cauliflower T/Cs (data not shown). Internal standard (IS; d_5-Fluoxetine) recoveries were low and variable (medium 17 ± 9; roots 34 ± 27; stem 28 ± 11; leaves 19 ± 14; curd 25 ± 17) as has been observed previously for some PPCPs in plant material (Boxall et al., 2006), but it was nonetheless possible to detect the IS in all exposed and non-exposed samples and thus to measure Fluoxetine, where present.

Total recovery (uncorrected for losses of IS) of applied Fluoxetine, including Fluoxetine remaining in the medium, ranged from only ~15% to 57%, with an average of 30%. Thus an average loss of 70% of the originally spiked Fluoxetine HCl had occurred. Although this may have been partly due to poor recoveries of Fluoxetine, this should have been accounted for by comparative losses of the added deuterated Fluoxetine HCl internal standard, against which the Fluoxetine concentrations were measured. It is more likely that a proportion of the losses were due to photodegradation of Fluoxetine HCl during the growth experiments. Fluoxetine HCl is a photolabile substance with a measured half-life of 55 h (rate constant = 0.0126 h^{-1}; Lam et al., 2005). Analysis of the extracts of *Brassicaceae* in pilot studies (Redshaw, 2008) for the known photoproducts of Fluoxetine HCl (cf. Lam et al., 2005) did not reveal these compounds (Redshaw, 2008), but the duration of the growth period in the present experiments (>23 half-lives) would be more than sufficient to account for the observed losses and might have resulted in mineralisation of any intermediate photoproducts. Plant metabolism and irreversible sorption to plant components may also have played a role in the low recoveries of Fluoxetine (Zebrowski et al., 2004).

Consideration of only the plant tissue sub-samples (i.e. excluding Fluoxetine remaining in the medium) showed total plant uptake ranging from 2.7% to 10.5% of the available burden, with an average of 8% (Table 3). Thus, plant uptake of Fluoxetine was apparently demonstrated. The confirmed presence of Fluoxetine HCl in all of the stems (mean 5 ± 2.4% of applied burden; $n = 5$) and some of the leaves (mean 3 ± 3.5% of applied burden; $n = 4$) also indicated that xenobiotic transport did occur under these experimental conditions in some of the experimental plants. It is clear that uptake to the leaves was not uniform in all experimental samples and no uptake to the curd was detected; nor was Fluoxetine detected in the roots. The data also show that significantly higher quantities of Fluoxetine remained in the medium when uptake did not proceed as far as the leaves (Fig. 2), with a larger proportion of Fluoxetine also remaining within the stems under these

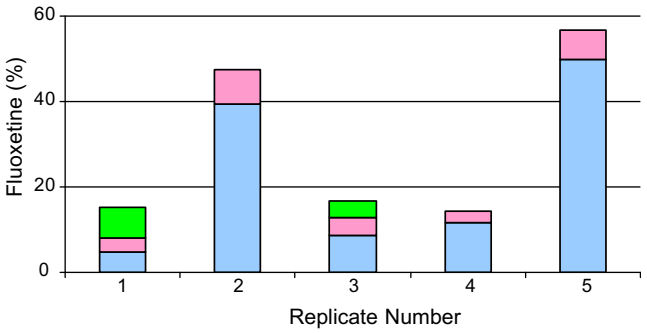


Fig. 2. Fluoxetine uptake in tissue culture samples from individual cauliflower plants. Percentage is expressed as % of total Fluoxetine added to each growth pot (9.8 μg). (■) Leaves; (■) stem; and (■) medium.

conditions. This supports the contention that transport occurred from the medium, through the roots and into the stem and leaves. The data in Fig. 2 also highlight the large losses of target compound when uptake to leaves did occur. These losses of Fluoxetine HCl, which appear to occur en route to the leaf, may be due to plant metabolism (Zebrowski et al., 2004).

Fluoxetine was not detected in the roots of any of the plants, which may at first seem surprising if transport of Fluoxetine HCl was *via* the roots to the stem, as proposed above. We feel the most likely explanation for this is that the root mass was so small, and hence the limit of detection (LOD) so high, that any Fluoxetine present was below the LOD. However, it is also possible that Fluoxetine was 'stored' in the leaves and stems, whereas in the root system the passage of Fluoxetine may have been transient, resulting in concentrations below the LOD (Collins et al., 2006). The low root concentrations may also be related to the temporal photodecomposition of Fluoxetine. As the exposure period increased through the growth phase there would have been greater photodecomposition and loss of Fluoxetine from the medium with time. Thus, at the beginning of the study when the Fluoxetine concentration was highest, translocation probably occurred from the roots to shoots. However, by the end of the study the concentration of Fluoxetine in the medium would have decreased (indeed had; Fig. 2) due to photodegradation. At this point, transfer to root tissues would have been correspondingly lower and by this time Fluoxetine originally present in the roots would have likely been translocated to the stems (Fig. 2).

Combining sub-samples of roots, or extending the growth period so that more substantial root systems are developed, might lead to the detection of Fluoxetine in the roots in future studies. Tissue cultures proved to be an easy and relatively quick way to screen for PPCP uptake in plants, and could be used in the future for routine screening, although extension of growth period to full maturity may be advisable. Indeed performing the same experiment with longer growth periods and sampling the plants at intermediate growth stages would be valuable. Sampling at various growth stages would also provide more data regarding the connection between lipid content and xenobiotic storage.

As an initial attempt to investigate the latter, a modified version of the Bligh and Dyer (1959) method for the extraction, purification and quantification of lipids was used to generate quantitative data on the amounts of lipid in cauliflower T/C stem and leaf samples (Table 4). Leaf lipid data (0.42% wet weight) were comparable with literature data for cauliflower florets stored at −85 °C (0.4% wet weight; Baardseth, 1977). The data showed that mean Fluoxetine concentrations in the stems (0.49 μg g^{-1} wet weight; 0.29 μg mg^{-1} lipid), were considerably higher than those in the leaves (0.26 μg g^{-1} wet weight; 0.03 μg g^{-1} lipid) although these differences were not statistically significant at the 95% confidence interval (*t*-test *P*-value = 0.08, 0.07, 0.17 for μg g^{-1}, μg mg^{-1} and % data, respectively). Although the average Fluoxetine concentrations were higher in the stem than the leaf samples, the average lipid concentrations were significantly higher in the leaf samples (stem 1.69; leaf 4.15 mg lipid g^{-1} wet weight; *t*-test *P*-value = 0.02). Thus Fluoxetine concentration does not appear to be directly associated with lipid contents in the plants. It is also possible that more extensive uptake to the leaves did occur, but that the extraction method was unable to recover Fluoxetine that was tightly bound.

Table 4
Lipid concentrations of cauliflower tissue culture leaf and stem sub-samples

	Lipid concentration (mg g^{-1} wet weight)	Lipid concentration (% wet weight)
Stem (n = 5)		
Mean	1.69	0.17
Standard deviation	0.92	0.09
% RSD	54.6	54.6
Leaves (n = 6)		
Mean	4.15	0.42
Standard deviation	1.63	0.16
% RSD	39.2	39.2

The preliminary finding herein, that Fluoxetine underwent uptake into cauliflower stem and leaves, extends current knowledge of the transport and fate of pharmaceuticals in the environment. During method development work for the present study, further evidence to support the translocation of Fluoxetine into other *Brassicaceae* tissues (*Brassicaceae Lepidium sativum* (cress)) was also found (Redshaw, 2008). On average 30% of applied burden was translocated to leaves (6 ± 3%), roots and stem (6 ± 2%) and associated with seed-casings (18 ± 4).

In other studies, antibiotics, including Trimethoprim used as a medicine for both animals and humans, were taken up by lettuce and carrots (ca. 6 ng g^{-1}) and the veterinary antibiotic Levamisole (ca. 170 ng g^{-1}) was taken up by lettuce, from unmanured soils spiked with 1 mg kg^{-1} of each substance (Boxall et al., 2006). Kumar et al. (2005) showed that between 0.2% and 1% of applied Chlortetracycline (2–17 ng fresh weight g^{-1}) was taken up from spiked and unspiked manured soils into corn (*Z. mays* L.), green onion (*Allium cepa* L.) and cabbage (*B. oleracea* L. Capitata group). Similarly Dolliver et al. (2007) showed that between 100 and 1200 ng dry weight g^{-1} of Sulfamethazine was taken up into corn (*Z. mays* L.), lettuce (*L. sativa* L.) and potato (*S. tuberosum* L.). After 45 days <0.1% of the amount applied to soil was accumulated.

Thus a small, but growing, body of evidence shows that some plants can take up some PPCPs. Further work must be completed before any major conclusions can be drawn, but the results highlight the need for further research, as suggested previously (Boxall et al., 2006).

Should plant uptake of pharmaceuticals prove to be a common phenomenon, further contamination risks arising from this should be assessed; such as the potential for transport along the food chain. In the case of plant uptake, plant species, soil type and environmental factors (e.g. soil moisture, pH, temperature), may influence uptake of xenobiotics.

3. Concluding remarks

In a simple preliminary uptake study with cauliflower grown in soil-free media, the pharmaceutical Fluoxetine was shown to be taken up from the medium and translocated to the stem in 5 of 5 plants and to the leaves in 2 of 4 replicates. Identification and quantification of Fluoxetine was made by HPLC–ESI–MS^n, including two stages of MS. Mean Fluoxetine HCl concentrations for stems were greater than for leaves, but this difference was found to be not statistically significant at the 95% confidence interval. No evidence of uptake to the most commonly eaten part of the plant, the curd, was found. Whilst the results suggest that Fluoxetine could be found in cauliflowers (and related *Brassicaceae*) grown on Fluoxetine-containing SS amended soils due to plant uptake, conclusions drawn from this experimental work should be treated as preliminary due to the limited environmentally relevant conditions, notably the lack of soil. Uptake from soil may be the same or may differ significantly from the mechanism identified herein. However, these results do suggest that further research into the uptake of pharmaceuticals into crops would be of value. With persistent compounds, such as some pharmaceuticals, there is a potential for accumulation within environments such as field soil to which SS is regularly added both as a disposal mechanism and as a fertiliser. When compounds undergo accumulation, the risk of transport to other environmental components becomes more

likely. From field soils these may include potential exposure to flora and fauna and possible bioaccumulation in terrestrial organisms and plants, including crops grown on the SS-treated soils, albeit at low concentrations.

4. Experimental

4.1. General experimental procedures

High performance liquid chromatography–mass spectrometry (HPLC–MS): all MS work carried out in this project used an electrospray interface fitted to a Finnigan MAT LCQ™ (ThermoFinnigan San Jose, CA, USA) quadrupole ion trap mass spectrometer. Instrument tuning and optimisation of mass calibration, was performed regularly throughout this project, using automatic calibration procedures and calibration solutions (caffeine, Sigma, St Louis, MO, USA; MRFA, Finnigan Mat, San Jose, CA, USA; Ultramark 1621, Lancaster Synthesis Inc., Widham, NH, USA; in MeOH: water: acetic acid (50:50:1 v/v/v)). High flow analysis required the coupling of an HPLC gradient pump (Dionex P580 quaternary pump) with an autosampler (5 μL injection volume; Dionex ASI-100 automated sample injector). Xcalibur 1.0 spl software (ThermoFinnigan) was used for data acquisition and processing. All high-flow work was performed under the following parameters: source voltage (+) 4.5 kV; capillary voltage (+) 0–50 V (set by auto tune function); capillary temperature 220 °C; nitrogen sheath gas flow rate 60 arbitrary units; auxiliary gas flow rate, 20 arbitrary units. Reverse phase separation was performed using a Phenomenex Gemini C18, 5 μm, 15 cm × 2.1 mm i.d column with aqueous (A: Chromasolv LC–MS grade water modified with 0.1% formic acid) and organic eluents (B: Chromasolv LC–MS grade ACN modified with 0.1% formic acid) under gradient conditions (20–100% B over 18 min, 5 min hold) at 0.2 mL min^{-1}.

Sigma–Aldrich was used as the source for acetonitrile (ACN) and methanol (MeOH) for LC–MS analysis (Chromasolv LC–MS grade, 99.9%). Formic acid (100% Aristar) was obtained from VWR, as was orthophosphoric acid (95% AnalaR BDH). Fluoxetine (*N*-methyl-3-phenyl-3[4′-trifluoromethyl-phenoxy]-propan-1-amine) was purchased as the HCl (>99% purity) from LCG Promochem. The internal standard, deuterated Fluoxetine (*N*-methyl-3-d5phenyl-3[4′-trifluoromethyl-phenoxy]-propan-1-amine) was obtained from Sigma–Aldrich as the HCl (1 mg mL^{-1} in MeOH, >98% D).

5. Source of biological materials

5.1. Cauliflower tissue culture, media preparation

The totipotent capacity of many plant cells lies at the basis of cell culture work. To take advantage of this capacity and to create clones from apical meristems, the growth medium for tissue culture must contain all the nutrients required, a carbon source, agar to solidify the medium and growth regulators, such as the plant hormone indole-3-acetic acid (IAA) and kinetin which promotes cell division. Murashige and Skoog (1962) medium, which was adapted for use with cauliflower floral meristems (50% strength with 8 g L^{-1} agar, 20 g L^{-1} sucrose, IAA 0.1 mg L^{-1}, kinetin 3.8 mg L^{-1}, adjusted to pH 5.8 with KOH), was used for the present work.

Media were sterilised by autoclaving (120 °C, 20 min) and then placed in a steamer (110 °C for 2 h) to liquefy. Prior to pouring, the medium was spiked with Fluoxetine HCl in MeOH (140 μg 500 mL^{-1} of medium) and stirred with a magnetic flea. Medium (35 mL) was then poured into plastic pots with lids and allowed to set in a laminar flow hood, resulting in a final concentration of 280 ng mL^{-1} (9.8 μg pot^{-1}). Equivalent blank pots, spiked with only MeOH, were also prepared.

5.2. Cauliflower tissue culture, tissue culturing

A cauliflower curd (Organic, Marks and Spencer Class 1) was aseptically dissected into approximately 1 g curd meristem sections (~4–5 mm diameter at base). Each explant was then weighed and the weight recorded. Explants were washed in 100% ethanol before being placed into beakers containing bleach solution (10% Marks and Spencer thick bleach with limescale control in deionised water) and a few drops of Tween (wetting agent) for sterilisation and shaken on a rotary table (5 min).

Tissue culturing was performed in a laminar flow hood where explants were removed from the bleach using forceps (heat sterilised), rinsed with deionised water and dried on non-fibrous tissue paper. These were then transplanted to Murashige and Skoog medium in airtight plastic pots. All pots were then placed into a growth cabinet at 20 ± 2 °C with a 12 h light/dark cycle (cool-white fluorescent light source; photosynthetic photon flux 130 ± 10 μmol m^{-2} s^{-1}). During the growth period the cauliflower plantlets were checked regularly for contamination or death and any infected samples were removed from the growth cabinet immediately. After 12 weeks growth all samples were stored at −80 °C.

5.3. Cauliflower tissue culture, harvest

Samples for harvest were selected at random (5 blank and 5 spiked) from the freezer. The frozen plants were then cut into sections using a scalpel. Meanwhile media and roots were allowed to defrost at room temperature before separating the roots from the medium. The T/C were sectioned to create sub-samples; curd, leaves, stem (including internode, node, stem and petiole), roots and media. All sub-samples were then washed in extraction solvent, and the washings added to the corresponding medium sample before weighing each sub-sample.

6. Experimental procedures

6.1. Cauliflower tissue culture, matrix effects

Cauliflower (Marks and Spencer Class 1; as sourced for T/C experiments) was extracted in the same manner as for T/C samples. Bulk extractions of curd, leaves and stems were performed using tandem SPE. Minor alterations were made to the extraction procedure which included; increasing volume of extraction solvent to 30 mL 20 g^{-1} wet weight of plant material (250 mL^{-1} for media); following drying samples were made up to 200 mL (4 × 50 mL portions Milli-Q) and re-filtered (Whatman No. 1) before undergoing SPE extraction. Single replicates of three different concentrations of ratio calibration samples (0.5, 2.5 and 7.5 μg mL^{-1} Fluoxetine HCl with 5 μg mL^{-1} IS) were prepared using the extracted cauliflower stem, curd and leaf matrices. Analysis was performed using SIM, as in the final T/C experiment. Data were then integrated and comparisons drawn between integrated data to ascertain whether matrix components were causing any interferences, such as ion suppression or enhancement.

6.2. Lipid extraction and quantification

The established Bligh and Dyer (1959) method for the extraction, purification and quantification of lipids were modified for use with cauliflower T/C stem and leaf samples. The Bligh and Dyer method is designed for use with 100 g samples containing ~80 ± 1% water and ~1% lipid. However, cauliflower T/C sub-samples are significantly lower in mass than this, and the cauliflower florets have a mean moisture content of ~91% and a lipid content

of ~0.4% wet weight (Baardseth, 1977). Solvent volumes were therefore altered to take account of the different tissue masses and average water content.

Cauliflower T/C was used as the sample source for this lipid work. The following method applies to a tissue mass of 1 g, therefore solvent volumes were altered to take account of the different masses of each of the 5 stem and 6 leaf samples. After sectioning the T/C into leaf and stem samples, they were homogenised using a pestle and mortar, transferred to a vial and the wet weight recorded.

Chloroform and MeOH (1.1375 and 2.275 mL g^{-1}) were then added to vials, to give a chloroform:MeOH:H_2O of 1:2:0.8, and shaken for 2 min. Additional chloroform was then added (1.1375 mL g^{-1}) and shaken for 30 s, followed by Milli-Q (1.1375 mL g^{-1}) and an additional 30 s shaking (chloroform:MeOH:H_2O of 2:2:1.8). A Büchner funnel, lined with filter paper (Whatman No. 1), operated with slight suction was used to filter and transfer extracts to clean graduated vials. The volume of the chloroform layer, which contained the lipids, was then recorded after complete separation and clarification. An aliquot (typically 500 μL) of the chloroform layer was then transferred to pre-weighed vial, and dried using a gentle stream of nitrogen at 40 °C, prior to re-weighing. To confirm that all non-lipid material had been removed, chloroform (~300 μL) was added to each dry vial to ensure that no insoluble material remained. The lipid content of each sample was then calculated (total lipid mass = (weight of lipid in aliquot × volume of chloroform layer)/volume of aliquot). This was then used to determine average lipid contents for leaves and stems, which was in turn used to calculate lipid quantities in exposed T/C samples.

6.3. Sample preparation and analysis

Sub-sectioned plant tissues were crushed with the extraction solvent (ACN with 1% formic acid; 5 mL) and sand (~1 g) using a pestle and mortar and transferred to centrifuge tubes, to which IS was added (2 μg). Sonication (15 min) was then followed by centrifugation (1500 rpm, 15 min). This process was repeated in triplicate and the supernatants combined and filtered (Whatman No. 1). The samples were then air dried to reduce the organic content to 4 mL, and then made up to 200 mL with Milli-Q and H_3PO_4 (40 μL).

These samples were then subjected to solid phase extraction (SPE). One Phenomenex Strata-SAX (Tri-func SAX, 500 mg^2 g^{-1}, 500 mg/3 mL) and one Phenomenex Strata-X (polymeric sorbent, 500 mg/3 mL) SPE cartridge were used per sample. All cartridges were conditioned and equilibrated with MeOH (3 ml, Chromasolv LC/MS grade) and Milli-Q (3 mL) respectively. The cartridges were then placed in tandem (SAX cartridge on top), and the samples were loaded. Once loaded the SAX cartridges were removed. The remaining Strata-X cartridges were washed with 50% MeOH: 50% Milli-Q (v/v; 2 × 3 mL), which was followed by vacuum drying (5 min). The cartridges were eluted with MeOH modified with 1% formic acid (6 mL). Eluted samples were collected in vials and then underwent blow-down to dryness (N_2, BOC) and were stored at 4 °C in the absence of light. An IST VacMaster and Vacuubrand vacuum pump were used to pass the solvents through the cartridges at a flow rate of approximately 5 mL min^{-1}.

Just prior to analysis (HPLC–ESI(+)–MS^n) dried samples were reconstituted with ACN (200 μL) of which 100 μL were removed to an autosampler vial along with Milli-Q (100 μL modified with 0.2% formic acid). Samples were initially analysed using SIM for Fluoxetine (m/z 310.0) and d_5-Fluoxetine (m/z 315.0), for the generation of quantitative data. SRM was then used to confirm the identity of peaks with full MS m/z 310.0 as Fluoxetine (m/z transition: 310.0–147.9).

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