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Novel Approach to the Extraction of Herbicides and Their Metabolites from Plant Tissues[1]

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A pressure extraction technique was developed and used to extract the herbicide atrazine and its metabolites from plant tissues of bean, soybean, and canola. Single leaves were placed in a pressure chamber, and plant fluids were expressed under 0-6.2 MPa of applied pressure through and collected from the leaf vascular system. Up to 98% of the total tissue fluid was extracted, and the procedure provided "clean" samples that were injected directly into an HPLC for analysis. Pressure-concentration release curves for atrazine and metabolites, and freeze-thaw treatment to disrupt membrane integrity, allowed interpretation of herbicide compartmentalization and metabolism within tissues. The pressure extraction procedure should prove to be very useful in the study of plant-herbicide relations.

The "Scholander-Hammel" pressure chamber has been extensively used to determine a variety of plant water relations parameters including tissue osmotic potential, tissue water potential, cell wall bulk modulus of elasticity, and amounts of bound and osmotically active water (Ritchie and Hinckley, 1975; Tyree et al., 1973; Hellkivist et al., 1974). Stroshine et al. (1979) has used the technique to model water movement in wheat leaves. The expression of tissue fluid by applied pressure has also been used to collect sap for solute analysis; Ackerson (1982) collected fluid for abscisic acid analysis, and Hartung et al. (1988) investigated absicisic acive movement in water-stressed cotton leaves. Recently, Jachetta et al. (1986a) have used a "pressure dehydration" technique to deduce the origin of expressed fluid. They distinguished three origins of sap as pressure was increased over small, 0.02-0.04 MPa, pressure intervals between 0.0 and 0.5 MPa. These sap fractions were sequentially released, and their origins were petiole-main vien fraction, minor vien-cell wall fraction, and a mixed fraction comprised of a decreasing minor vien-cell wall component containing increasing amounts of plasma membrane filtered symplastic fluid. Further studies by Jachetta et al. (1986b) determined the transport and distribution pattern of shoot-fed herbicides, atrazine and glyphosate, within detached sunflower leaves. Their results confirmed that atrazine movement followed the apoplastic pattern (Ashton and Crafts, 1973) whereas glyphosate movement was mainly via a symplastic pattern (Dewey and Appleby, 1983; Gougler and Geiger, 1981). Jachetta et al. (1986a) indicated the potential usefulness of the pressure dehydration method for the study of sub-

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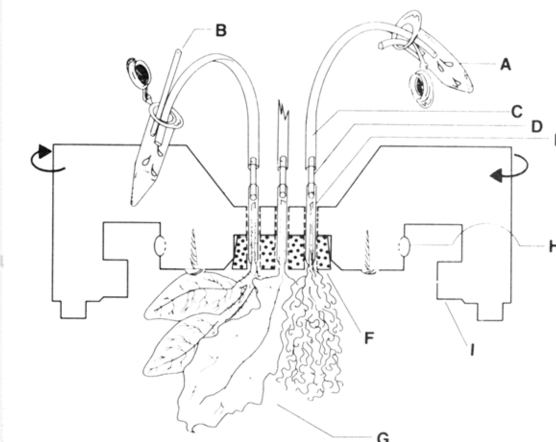

Figure 1. Pressure extraction apparatus for extraction of atrazine and metabolites residues from plant tissue. Pressure chamber and line drawing of cross-sectional view of chamber cap: A, fluid collection vial with vial cap; B, fluid collection vial vent through cap; C, clear tygon tubing; D, glass capillary tube; E, clear heat shrink tubin; F, No. 7 Neoprene rubber stopper; G, plant sample; H, rubber O-ring to seal cap to chamber; I, lip to lock cap to chamber.

stances that translocate via the apoplastic route.

The objective of this study was to extend the use of the pressure dehydration technique to whole plant systems in order to develop an alternative method to chemical extraction in routine tissue analysis for residues of atrazine and its metabolites. A much larger range of applied pressures was used in order to perform material balance calculations and to determine whether the extraction patterns of atrazine and metabolites could be used to study compartmentalization and metabolism.

MATERIALS AND METHODS

Plant Treatment. *Vicia faba* Seafarer (bean), *Brassica napus* ATR Tower (canola), and *Glycine max* (L.) Merr Maple Arrow (soybean) were grown in silica quartz sand culture in Ray Leach containers (Canby, OR) under a 16-h photoperiod of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ and day and night temperatures of 25 and 20 °C, respectively. Plants were fertilized with Long Ashton nutrient solution (Hewitt, 1966) and grown for either 20 or 50 days prior to use in uptake experiments. The herbicide uptake studies were carried out with "cold" and radiolabeled atrazine. In both cases plant root systems were partially immersed in treatment solutions comprised of Long Ashton nutrient solution containing 10 ppm atrazine. In the case of "cold" atrazine application, treatment times of 3, 24, or 48 h were used after which samples were removed for extraction and residue analyses. This experiment involved three replications each for bean, soybean, and canola. In the case of the [^{14}C]atrazine uptake study, three canola plants were treated with 500 mL of nutrient solution containing 10 ppm atrazine for 48 h and three leaf samples were removed from each plant for extraction and analysis. The uniformly ^{14}C ring-labeled atrazine (specific activity 49.4 mCi/mmol, purity 98%) used in this study was a gift from the Agricultural Division of Ciba Geigy Corp., Basel, Switzerland.

Pressure Extraction Procedure. A Model 1000 pressure chamber (PMS Instrument Co., Corvallis, OR) was used to extract fluid from plant tissue (Figure 1). The method described by Cheung et al. (1975) was used with the following modifications. The pressure chamber cap was modified to allow insertion of three samples into the chamber (Figure 1). Plants were hydrated by immersion of the root system in treatment solutions. The youngest fully expanded leaves with attached petiole were sampled after at least 90 min in the dark covered by a plastic bag. In the case of 24- and 48-h treatments, samples were taken predawn, following a dark period. Single leaves of canola and trifoliate petioles of soybean and bean were used. Bean samples also contained seed pods and developing seeds. The distal portion of the petiole, ca. 1.5 cm, was lightly crushed with a glass rod to expel fluid and then blotted dry prior to weighing for fresh weight and insertion into a heat shrink tubing collar (Figure 1E). This was required as heating fresh tissue produced a discoloration of the initial fraction of expressed fluid and a poor seal between the petiole and the heat shrink tubing collar due to moisture condensation between these surfaces. The heat shrink tubing was heated and shrunk onto the lightly crushed portion of the petiole and a glass capillary tube (Figure 1D,E) to form a sealed conducting system. The plant tissue and collar were inserted through a Neoprene rubber stopper (Figure 1F), which was sealed into the pressure chamber cap. Leaves were sealed in plastic bags in order to minimize water vapor loss in the pressure chamber. A fluid collection assembly (Figure 1A–D) was weighed and attached to the collar prior to application of pressure.

Pressure was applied to the sample in increments of 0.17 MPa up to 1.03 MPa, in increments of 0.35 MPa up to 2.07 MPa, and then in increments of 0.69 MPa up to 6.2 MPa. At each set pressure, plant fluid was collected in vials for 20 min; the bulk of the fluid extracted at each set pressure was collected within the first 10 min. After each 20-min period, extracted fluid was weighed and the pressure released to the previous balance pressure at a constant low rate of release (0.3 $MPa \cdot min^{-1}$) in order to avoid temperature-caused deterioration of tissues. Pressure was then increased slowly until fluid returned to the cut surface of the petiole; this pressure was recorded as the balancing pressure. The procedure was repeated up to the limit of the pressure chamber (6.2 MPa) and outside the range of physiologically interpretable pressures (Hellkivist et al., 1974). Collection vials were periodically changed to avoid overloading and air desiccation of extracted fluid and to fractionate the extract for herbicide analysis. The extracts were analyzed by gas chromatography (GC) and high-pressure liquid chromatography (HPLC).

Pressure–volume release curves describing the relationship between the inverse of the balancing pressure and the volume of fluid expressed were constructed and used to calculate several parameters important to an understanding of water partitioning within tissue. The original volume of osmotically active water (V_o) was obtained by extrapolating the straight-line portion of the curve to the abscissa. The "bound water" was calculated from the difference between V_o and the total volume of water in the tissue obtained by oven-drying. These parameters were calculated according to the theory and formula presented by Hellkivist et al. (1974) and Tyree et al. (1973).

Duplicate tissues samples were placed in plastic bags and frozen for at least 24 h at −18 °C in a laboratory refrigerator. Prior to extraction of previously frozen tissues, samples were thawed in their plastic bags and then prepared in a manner similar to that of fresh tissues.

Solvent Extraction. All leaf samples were initially pressure-extracted. Untreated control leaves were used to determine

Table I. Partitioning of Water within Fresh Tissue of Bean, Canola, and Soybean and Frozen-Thawed Tissue of Canola Determined from Pressure Extraction of Leaf Tissue[a]

plant variety	dry wt, g	total water in tissue, mL	% total water extr by pressure	osmotically active water,[b] mL/% total	bound water,[c] mL/% total
bean (fresh)	0.890 ± 0.238	5.341 ± 1.221	28.4 ± 12.3	2.16/40.4	3.18/59.4
soybean (fresh)	0.230 ± 0.031	0.632 ± 0.072	63.9 ± 6.9	0.54/85.4	0.09/14.2
canola (fresh)	0.131 ± 0.013	0.950 ± 0.110	72.6 ± 1.6	0.94/98.5	0.01/1.5
canola (frozen)	0.107 ± 0.012	0.798 ± 0.122	94.7 ± 0.7	>0.76/>95.2	<0.04/<5.0

[a] Mean of nine samples ± 95% confidence interval. [b] Estimated from pressure–volume curves. [c] Calculated from total water in tissue minus estimated osmotically active water.

background levels for all analyses. In the case of the "cold" atrazine uptake study with canola, soybean, and bean, aliquots of the pressure extracts were directly analyzed by HPLC without any sample cleanup. In the case of the [^{14}C]atrazine uptake studies, after pressure extraction the leaves were blended at a high speed with methanol (1:100, w/v) for 15 min. The mixture was filtered under suction and the sample residue washed with methanol. The procedure was repeated two more times. Further blending of the insoluble leaf material with methanol did not result in any measurable extractable ^{14}C. The insoluble leaf sample containing ^{14}C was dried and was then combusted to $^{14}CO_2$ to determine the total nonextractable ^{14}C. The methanol and pressure extracts were analyzed as described below. Thus, the total radioactivity was partitioned into pressure-extractable, methanol-extractable, and unextractable residues.

An aliquot of the pressure extract was taken in a round-bottom flask and evaporated just to dryness at room temperature with a gentle stream of dry nitrogen. The material was dissolved in methanol. Aliquots of this solution and the methanol extract containing ^{14}C residue were analyzed directly by GC while another portion was methylated with diazomethane prior to GC analyses.

Determination of Radioactivity. Combustion of the extracted leaf tissue was done in a Packard sample oxidizer, Model 306, to produce $^{14}CO_2$. The $^{14}CO_2$ was absorbed in and admixed with an appropriate volume of oxisorb and oxiprep (Packard Instrument of Canada, Ltd.). Aliquots of the extracts or solutions containing ^{14}C residues were analyzed in a Beckman series 8000 liquid scintillation spectrometer, using an external standard and correcting the data for quenching.

GC. The gas chromatograph was a Varian Model 6000 fitted with a thermionic nitrogen-specific detector. The capillary column was a 15 mm × 0.324 mm (i.d.) megabase column coated with DB-5 (1.5 μm). The oven temperature was programmed at 1.5 °C·min^{-1} from 180 to 220 °C and held at the latter temperature for 30 min. The detector and injector port temperatures were maintained at 290 and 190 °C, respectively. Helium was used as carrier gas at a flow rate of 15 mL·min^{-1}.

HPLC. The HPLC measurements were carried out on a Varian Model Vista 5500 chromatograph equipped with a Varian variable UV200 detector. The wavelength selected for all measurements was 254 mm. The stainless steel column (25 cm × 4.6 mm (i.d.)) was packed with Partisil 10 ODS-3 particle size 10 μm and was pretested by the manufacturer. The mobile phase used was methanol–water (60:40) at a flow rate of 1.5 mL·min^{-1}.

Confirmation of Atrazine and Metabolites. The identity of the compounds was confirmed by comparing the retention times with those of authentic samples and by cochromatography.

RESULTS AND DISCUSSION

Fluid Extraction from Plant Tissues. Fluids were pressure-extracted from all tissues tested. Fluid extraction from fresh tissues occurred between set pressures of 0.17 to 6.2 MPa, and the majority of extraction was complete by set pressures of 2.76 MPa. The maximum rate of fluid extraction in fresh tissue occurred between 1.38 and 2.07 MPa. In 50-day-old plants, 72.6% of total tissue water was pressure-extracted from fresh canola leaves, and in the atrazine uptake experiments with 20-day-old canola leaves, 76.8% of total tissue water was extracted (Table I).

The pressure–volume release curves for fresh tissue of bean, canola, and soybean were typical in that the relationship between the inverse of the balancing pressure plotted against the volume of fluid expressed was nonlinear

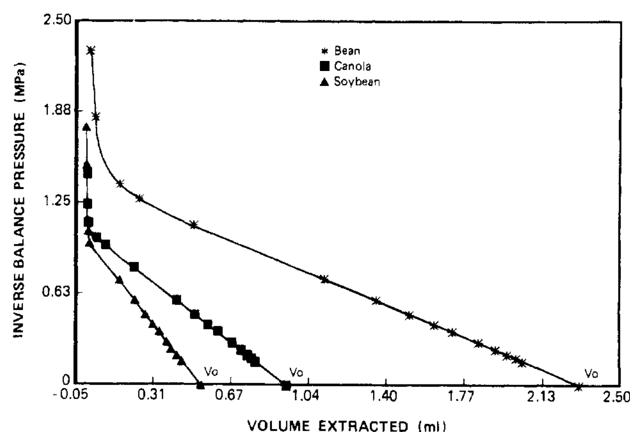

Figure 2. Pressure–volume release curve for bean, soybean, and canola leaf tissues. Data points represent the mean of nine samples. V_o = estimated original volume of osmotically active water.

(Figure 2). Inflection points were noted between 1.03 and 1.38 MPa, indicating that cell turgor pressure had gone to zero and the linear portion of these curves was extrapolated to the X-axis for estimation of the amount of ostomically active water (Table I). The amounts of pressure-extractable fluid and the estimated bound water varied among plant species (Table I) as was previously reported in other studies (Tyree et al., 1974). In bean samples, which contained seed pods and developing seeds, 59% of the total tissue water was estimated to be bound water, whereas only 2 and 14% were bound in canola and soybean, respectively. In fresh canola samples, 98% of the water was osmotically active water whereas only 85 and 40% were osmotically active in soybean and bean (Table I). Bound water, as calculated from pressure–volume release curves, is largely considered to be apoplastic, and the variation among species reflects differences in water sequestered in the cell walls or cell interstices. This water is held by strong retentive forces associated with capillarity and polymolecular binding to cell wall components such as pectins and hemicelluloses and would require impractically high pressures for the pressure extraction assembly: greater than 30 MPa for release (Hellkivist et al., 1974). The values presented here for amounts of bound versus osmotically active water agree with a range of values presented by Hellkivist et al. (1974) and Cortes and Sinclair (1985).

In frozen–thawed canola tissue the majority of fluid extraction occurred between 0.17 and 0.35 MPa, and very little additional fluid extraction occurred after 1.03 MPa. Therefore, the frozen–thawed canola had an atypical pressure volume release curve, and osmotically active and bound amounts of water were not calculated. The freeze–thaw treatment increased pressure extraction of water to 94.7% of total tissue water in canola leaves. This increase, 22.1% of total tissue water, must have originated from the osmotically active water in the tissue as fresh samples contained an estimated 98.5% osmotically active

Table II. Concentration of Atrazine and Dealkylated Atrazines in Pressure-Extracted Fluid and Tissue of Bean, Soybean, and Canola after 48-h Uptake of 10 ppm Atrazine Solution

plant variety	compd	total mole atrazine equiv, nmol·g^{-1} dry wt	% total mole atrazine equiv	estd tissue concn, ppm, μg·g^{-1} dry wt	ppm atrazine equiv, μg·g^{-1} extr fluid
bean	atrazine		50.0 ± 14.5[b]	18.9 ± 13.4[b]	8.2 ± 2.0[b]
	de-E At		24.9 ± 11.1	7.6 ± 0.2	3.6 ± 1.9
	de-Pt At		25.1 ± 12.1	7.5 ± 4.5	3.6 ± 3.2
	total[a]	171.2 ± 77.2[b]		36.9 ± 16.6	16.7 ± 6.8
soybean	atrazine		22.1 ± 9.2	9.9 ± 4.9	3.9 ± 1.5
	de-E At		77.9 ± 9.2	30.9 ± 12.2	12.5 ± 5.1
	de-P At		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	total[a]	211.4 ± 70.8		45.6 ± 15.3	18.3 ± 5.9
canola	atrazine		82.0 ± 1.7	37.4 ± 8.0	6.9 ± 1.4
	de-E At		14.5 ± 0.7	5.7 ± 1.1	1.1 ± 0.2
	de-P At		3.5 ± 1.6	1.3 ± 0.4	0.3 ± 0.1
	total[a]	211.8 ± 42.8		45.6 ± 9.2	8.4 ± 1.6

[a] Totals calculated after each metabolite was converted to mole atrazine equivalence. [b] Mean ± 95% confidence interval, $n = 3$.

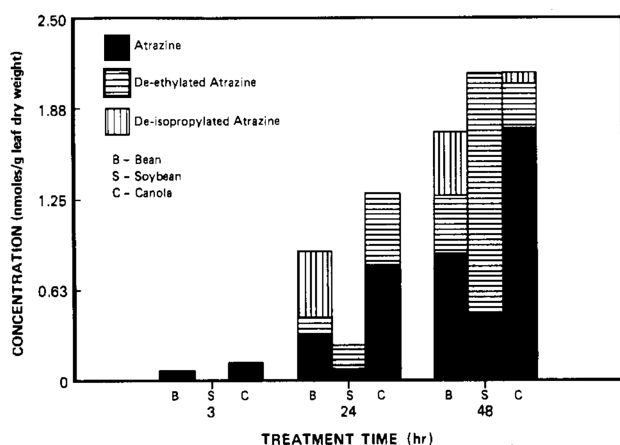

Figure 3. Concentration of atrazine and metabolites in leaves of bean, soybean, and canola determined by direct injection of pressure-extracted fluids into an HPLC. Mean of three samples.

water (Table I). Therefore, the freeze–thaw treatment released symplastic water that was not extractable from fresh tissue by the pressure extraction procedure.

HPLC Analysis of Pressure-Extracted Fluid. Extracted fluids of bean, soybean, and canola, injected directly into the HPLC, had significant levels of atrazine and dealkylated metabolites. However, no hydroxylated atrazine was detected in these extracts (Figure 3). Extracted fluids had greater levels of atrazine and dealkylated atrazine metabolites as treatment times increased. Differences in the proportion of atrazine and metabolites in the extracted fluids were noted (Figure 3). For example, in the 48-h treatment, 78% of the total atrazine mole equivalence (based on the total moles of all detectable *s*-triazine compounds in the extracted fluid) in soybean extracts was deethylated whereas in canola only 15% was deethylated (Table II). Soybean and canola pressure extracts contained less than 5% deisopropylated atrazine whereas in bean extracts 25% was deisopropylated. As treatment solution contained only atrazine and no metabolites, these differences represent different plant metabolisms and indicate the potential usefulness of the technique for herbicide screening and metabolism studies.

Levels of total *s*-triazines in leaf tissue increased with treatment time (Figure 3); however, the rates of uptake varied among the species. Rates were determined by HPLC analysis of pressure extracts and calculated based on the hours of light treatment as atrazine moves in the transpirational stream (Smith and Buccholtz, 1964). Rates between 3–24- and 24–48-h treatment times remained the same for bean (5.1 nmol·g^{-1}·h^{-1}), increased for soybean (1.4–11.7 nmol·g^{-1}·h^{-1}), and declined in canola (7.3–5.1 nmol·g^{-1}·h^{-1}). The drop in canola uptake rate after 24 h is explained by a reduction in transpiration caused by atrazine-induced stomatal closure (Smith and Buccholtz, 1964). Therefore, the pressure extraction technique coupled with direct analysis of extracts by HPLC could be used to determine varietal differences in uptake rates, treatment effects on uptake, and uptake characteristics for various test agrochemicals.

Table III. Distribution of ^{14}C Label in Fresh and Frozen Leaves of 20-Day-Old Canola Plants after 48-h Root Treatment with Labeled 10 ppm Atrazine Solution

leaf treatment	sample type[a]: pressure extr	methanol extr	leaf res	total
	(a) Dpm × 10^4 g^{-1} Dry Weight of Leaf Residue			
fresh[b]	2.34[b]	19.31[a]	1.21[a]	22.86[a]
frozen	7.16[a]	12.94[b]	1.07[a]	21.17[a]
	(b) Percent of Total Dpm			
fresh	10.3[b]	84.5[a]	5.2[a]	
frozen	33.8[a]	61.2[b]	5.1[a]	

[a] All leaves were pressure-extracted and then methanol-extracted. [b] Mean separation within a column by Duncan's multiple-range test, $P = 0.5$, reps = 3.

^{14}C Analysis of Pressure-Extracted Fluid in Canola. Leaf tissues of canola contained 22.86 × 10^4 dpm·g^{-1} leaf residue dry weight after a 48-h root treatment with 10 ppm [^{14}C]atrazine solution containing 0.207 × 10^4 dpm·mL^{-1} of treatment solution (Table III). Of the total radioactivity in fresh leaves, 10, 85, and 5% were found in the pressure-extractable, methanol-extractable, and unextractable leaf residue portions of the leaf, respectively (Table III). Thus, a relatively small portion of total leaf triazines was associated with osmotically active water and therefore pressure-extractable. A much larger percentage was released by methanol extraction as expected due to the greater solubility of atrazine in methanol: 33 ppm solubility in water versus 18000 ppm solubility in methanol. Freezing and then thawing the tissue before pressure extraction resulted in a significant increase in the amount of radioactivity in pressure extracts with a concomitant decrease in methanol extracts and no change in the unextractable residues. Freeze–thaw treatment did not alter the total extractable or total amount of radioactivity found in leaf tissues (Table III). The physiochemical events associated with freeze–thaw in tender canola leaves were expected to disrupt membrane integrity (Stoponkus and Wiest, 1981) and hence cellular compartmentalization of *s*-triazines. Although the extent of membrane lysis or loss of semipermeability was not directly measured, the comparison of radioactivity in extracts of fresh and frozen–thawed canola indicates that membrane integredity

Table IV. Concentration of Atrazine and Its Metabolites in Pressure Extracts and Methanol Extracts of Fresh and Frozen Canola Leaves from 20-Day-Old Plants after a 48-h Root Treatment with 10 ppm Atrazine Solution As Determined by GC Analysis

leaf treatment	compound	sample type: pressure extr	sample type: methanol extr	sample type: total extr	pressure vs methanol
(a) Micromoles per Gram Dry Weight of Leaf Residue					
fresh	atrazine[c]	0.205^{b}	1.601^{a}	1.807^{b}	b, a[a]
	OH-At	0.002^{a}	0.180^{a}	0.182^{a}	b, a
	de-E OH-At	0.019^{b}	0.014^{a}	0.033^{b}	a, a
	de-P OH-At	0.014^{b}	0.124^{a}	0.138^{b}	b, a
	de-E At	0.383^{b}	0.998^{a}	1.381^{a}	b, a
	de-P At	0.006^{b}	0.021^{a}	0.027^{a}	a, a
	total At[b]	0.630^{b}	2.938^{a}	3.569^{b}	b, a
frozen	atrazine	0.554^{a}	1.474^{a}	2.027^{a}	b, a
	OH-At	0.065^{a}	0.094^{a}	0.159^{a}	a, a
	de-E OH-At	0.121^{a}	0.032^{a}	0.153^{a}	a, a
	de-P OH-At	0.287^{a}	0.046^{a}	0.335^{a}	a, a
	de-E At	0.957^{a}	0.487^{b}	1.444^{a}	a, a
	de-P At	0.095^{a}	0.020^{a}	0.115^{a}	a, a
	total At[b]	2.079^{a}	2.154^{a}	4.232^{a}	a, a
(b) Percent of Total Atrazine Mole Equivalence for the Sample					
fresh	atrazine[c]	5.8^{b}	45.0^{a}	50.9^{a}	b, a[a]
	OH-At	0.1^{a}	5.2^{a}	5.3^{a}	b, a
	de-E OH-At	0.5^{a}	0.3^{a}	0.8^{b}	a, a
	de-P OH-At	0.4^{b}	3.5^{a}	3.8^{a}	b, a
	de-E At	10.9^{b}	27.6^{a}	38.5^{a}	b, a
	de-P At	0.7^{a}	0.5^{a}	0.7^{b}	a, a
	total At[b]	17.9^{b}	82.1^{a}		
frozen	atrazine	13.3^{a}	34.7^{b}	48.0^{a}	b, a
	OH-At	1.5^{a}	2.2^{a}	3.7^{a}	a, a
	de-E OH-At	2.9^{a}	0.8^{a}	3.7^{a}	a, a
	de-P OH-At	6.8^{a}	1.2^{a}	7.9^{a}	a, b
	de-E At	22.6^{a}	11.4^{b}	33.9^{a}	a, b
	de-P At	2.2^{a}	0.5^{a}	2.7^{a}	a, a
	total At[b]	49.3^{a}	50.7^{b}		a, a

[a] Mean separation between pressure and methanol extract by Duncan's multiple-range test for each compound, $P = 0.05$, reps = 3. [b] Total moles of atrazine equivalence determined from analysis of metabolites. [c] Mean separation within a column and compound by Duncan's multiple-range test, $P = 0.05$, reps = 3.

was disrupted. Freeze–thaw treatment resulted in a 200% increase in pressure-extractable *s*-triazines. As 60% of total *s*-triazines remained methanol-extractable after the freeze–thaw treatment, it would require very careful experimentation to relate pressure-extractable concentrations to total concentrations at target sites—high-affinity binding sites such as the chloroplast thykaloid membrane polypeptide of the photosystem II complex (Mullet and Arntzen, 1981). However, as equilibrium conditions should be established, pressure extracts could be used as valuable indicators of atrazine partitioning into the lipophilic phase of the tissue and possibly binding at target sites. Previous reports, Izawa and Good (1965), Price and Balke (1983), and Jachetta et al. (1986b), indicate that the lipophilic phase of plant tissue represents a significant reservoir for atrazine and its nonpolar metabolites.

GC Analysis of Pressure-Extracted Fluid in Canola. GC analysis indicated the presence of deethylated and deisopropylated and hydroxy metabolites of atrazine in both pressure and methanol extracts (Table IV). Significantly greater amounts of all metabolites except deethylated hydroxyatrazine and deisopropylated atrazine were found in methanol extracts as compared to pressure extracts. All atrazine metabolites in the pressure extract, except hydroxyatrazine, were significantly increased due to freeze–thaw. The order of increase was deethylated atrazine > atrazine > deisopropylated hydroxyatrazine ≫ other metabolites (Table IV). This order may reflect the degree of compartmentalization within the cell, the affinity for binding sites, and the degree of partitioning into the lipophilic phase of the plant tissue. In frozen–thawed tissues, there was no significant difference in total atrazine mole equivalence between pressure and methanol extracts (Table IV). In both fresh and frozen–thawed tissues, approximately 50% of the total atrazine mole equivalence was in the form of atrazine and 36% was found as deethylated atrazine (Table IV). These findings agree with other studies of atrazine metabolism. For example, Raynaud et al. (1985) found that 23% of total *s*-triazines was deethylated and 45% was atrazine in root-treated barley.

Pressure–Volume Release Curve Analogy. Pressure release curves of radiolabeled materials and of atrazine and metabolites in fresh leaf samples were similar in form to that found for water, and therefore the pressure–volume analogy was applied to estimate amounts associated with osmotically active water and amounts bound or not associated with osmotically active water. The radioactivity and total atrazine mole equivalence associated with osmotically active water were estimated to be 3.41×10^4 dpm·g^{-1} dry weight of leaf residue and 0.91 μmol·g^{-1} dry weight of leaf tissue (Table V). These exceeded the amounts that were pressure-extracted from fresh tissue (2.34×10^4 dpm·g^{-1} and 0.630 μmol·g^{-1}; Table III) as expected. The amount of radioactivity in the methanol extract plus the leaf residue (20.52×10^4 dpm·g^{-1}; Table III) was statistically similar to the bound radioactivity (19.46×10^4 dpm·g^{-1}; Table V) estimated from the pressure–volume analogy. Also, estimates of bound atrazine mole equivalence (2.660 μmol·g^{-1}; Table V) were statistically similar to the values in the methanol extracts (2.93 μmol·g^{-1}; Table IV). These estimates of bound *s*-triazine agree with those *s*-triazine concentrations determined by methanol extraction values with a mean conversion of 1.093 (atrazine metabolite analysis) and 1.056 (^{14}C analysis). Therefore, the application of the pressure–volume analogy, applied to these *s*-triazines, may refer to partitioning into the aqueous phase (estimated as osmotically active water) and into the lipophilic phase (estimated as bound and approximately equal to amounts methanol-extractable after pressure extraction). With the proper calibration, pressure extract analysis may provide insight into partitioning of *s*-triazines in plant tissue and suggest the possibility that only pressure extract data need be measured in order to estimate traditionally determined methanol extract data.

Elution Patterns of Atrazine and Metabolites. Although plots of concentration based on volume of extracted fluid indicate differences in release patterns among the metabolites, all metabolite patterns followed the same sequence (Figure 4). That is, an apparent plateau of concentration between 0 and 0.6 MPa, followed by a zone of transition between 0.6 and 1.0 MPa to a second plateau (0.9–1.2 MPa) which stabilized, except for the deisopropylated metabolites, between 1.5 and 4.0 MPa (Figure 4). Jachetta et al. (1986a) compared the osmolality of expressed sap to the concentration of a strictly apoplastic dye to indicate that the order of sap origin was the petiole midrib, the minor viens and cell wall, and mixed cell wall with increasing amounts of membrane-filtered symplastic origins. If this order of sap origin is applied to our data and we assume that plateaus in sap concentration during elution represent compartments, then the pattern of elution can be used to deduce the locations of atrazine and each metabolite, the site of metabolism, the form during transport, and membrane permeabilities. For example, hydroxyatrazine has its greatest concentrations in extracellular or apoplastic compartments and undetectable amounts in the symplast (Figure 4). As transport of *s*-

Table V. Partitioning of Radiolabel, Atrazine, and Its Metabolites in Fresh Canola Leaves Calculated from Pressure–Volume Release Analogy

	total tissue content[a]	osmotically active[b]	total bound[c]	leaf res
water, mL	2.03 ± 0.18	1.91 ± 0.25	0.13 ± 0.28	
radiolabel, dpm × $10^{-4}\cdot g^{-1}$ dry wt leaf residue	22.86 ± 2.86	3.41 ± 0.48	19.45 ± 2.58	1.21 ± 0.23
Atrazine and Metabolites ($\mu mol\cdot g^{-1}$ Dry Weight of Leaf Residue)				
atrazine	1.81 ± 0.30	0.28 ± 0.08	1.52 ± 0.30	
OH-At	0.18 ± 0.06	0.02 ± 0.01	0.18 ± 0.06	
de-E OH-At	0.04 ± 0.04	0.03 ± 0.02	0.02 ± 0.03	
de-P OH-At	0.14 ± 0.15	0.02 ± 0.03	0.12 ± 0.13	
de-E At	1.38 ± 0.45	0.57 ± 0.06	0.82 ± 0.40	
de-P At	0.03 ± 0.04	0.01 ± 0.02	0.02 ± 0.03	
total At[d]	3.57 ± 0.80	0.91 ± 0.15	2.66 ± 0.66	
Atrazine and Metabolite (ppm, $\mu g\cdot g^{-1}$ Water in Leaf Tissue)				
atrazine	25.71 ± 4.26	3.97 ± 1.14	21.59 ± 4.26	
OH-At	2.34 ± 0.78	0.26 ± 0.13	2.34 ± 0.78	
de-E OH-At	0.44 ± 0.44	0.33 ± 0.22	0.22 ± 0.33	
de-P OH-At	1.43 ± 1.53	0.20 ± 0.31	1.23 ± 1.32	
de-E At	17.05 ± 5.56	7.04 ± 0.74	10.13 ± 4.94	
de-P At	0.34 ± 0.46	0.11 ± 0.23	0.23 ± 0.34	
total At[d]	50.70 ± 11.36	12.92 ± 2.13	37.78 ± 9.37	

[a] Sum of pressure extract, methanol extract, and leaf residue if applicable. [b] Estimated from pressure–volume curve. [c] By subtraction, total – osmotically active. [d] Total moles of atrazine equivalence.

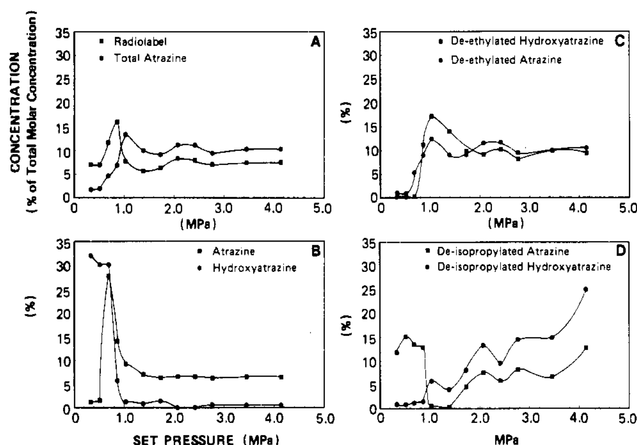

Figure 4. Elution patterns of radioactivity and atrazine and metabolites pressure-extracted from leaves of canola. Each data point is the mean of three.

triazines is apoplastic (Ashton and Crafts, 1973) and no hydroxyatrazine was found in the treatment solution, it is likely that atrazine was metabolized to hydroxyatrazine in the root system, that a portion of the hydroxyatrazine was transported to the shoot, and that a relatively high membrane reflection coefficient to hydroxyatrazine reduced its uptake into the leaf symplast. In contrast, high symplastic atrazine concentrations are shown in its elution curve, and freeze–thaw significantly increased the pressure extraction of atrazine. Therefore, atrazine has a relatively low membrane reflection coefficient. These conclusions are supported by Darmstadt et al. (1984) as they also found considerably higher membrane permeabilities for atrazine as compared to hydroxyatrazine in permeation experiments with excised root tissue of corn. They attributed the low membrane permeability for hydroxyatrazine to its low partition coefficient and low lipid solubility (Price, 1982).

As large amounts of these *s*-triazines remain in the tissue after pressure extraction (Table IV), the pressure extraction technique cannot be used to extract all of the *s*-triazines. Only a portion of the normally nonpressure-extractable *s*-triazines become pressure-extractable after freeze–thaw treatment while a portion still remains methanol-extractable. This may represent compartmentalization within the symplast due to variable partitioning of these materials in the lipophilic phase or binding to sites of differing affinities. For example, Stemler and Murphy (1984) have determined that maize chloroplast have both high- and low-affinity binding sites for atrazine, and Izawa and Good (1965) found reversibly and irreversibly bound atrazine concentrations during absorption studies of spinach chloroplasts. Therefore, these techniques allow parameterization of atrazine and metabolite concentrations within the leaf. For example, the compartmentalization of total *s*-triazines can be calculated from ^{14}C data and elution data to be associated with osmotically active water (petiole midrib, 0.4%; minor vien cell wall, 1.9%; symplast, 8.0%), partitioned into the lipophilic phase and bound to sites of differing affinities (lower level of partitioning or binding affinity, 32.8%; higher level of partitioning or binding affinity, 51.7%), and bound to tissue residue or unextractable (5.2%).

It is interesting to note that the ^{14}C elution pattern differs from the pattern for total atrazine mole equivalence in that ^{14}C apoplastic portions were much greater than total atrazine apoplastic portions (Figure 4). This suggests that other sources of ^{14}C were not analyzed in the GC analysis and also that these other metabolites appear mainly in the apoplastic system. Other than hydroxylated or dealkylated metabolites, Raynaud et al. (1985) suggest from their analysis of atrazine metabolism in barley that these other metabolites may be conjugated derivatives of atrazine.

Accumulation of Atrazine against a Concentration Gradient. Based on concentrations found in pressure extracts of the 48-h treatment, the total atrazine ppm equivalence (micrograms per gram dry weight of tissue) in leaf tissues was similar for bean, soybean, and canola tissues: 36.9, 45.6, and 45.6 ppm, respectively (Table II). The total atrazine ppm equivalences in the pressure-extracted aqueous fluids of bean, soybean, and canola were 16.7, 18.3, and 8.4 ppm (microgram per gram of pressure extract). These values were less than the water solubility of atrazine (33 ppm) and either similar to or greater than the total atrazine ppm equivalence of the treatment solution as measured at the end of the 48-h treatment, 10.1 ppm. In canola, *s*-triazines in the aqueous pressure-extractable portion of the leaf only represent approximately

10% of the total leaf *s*-triazines as determined by ^{14}C analysis (Table III). Therefore, the data indicate that atrazine was accumulated against a concentration gradient as reported previously for excised roots of velvet leaf (Price and balke, 1983) and detached leaves of sunflower (Jachetta et al., 1986b). Pressure extract studies including the analysis of the whole plant system would allow investigation of plant *s*-triazine allocation and identification of the sites of accumulation. In addition, measured levels of total atrazine mole equivalence, determined by GC analysis, represent a total atrazine concentration of 8.9 and 30.1 ppm (micrograms of atrazine per gram of water in leaf) in pressure extracts and 41.7 and 31.3 ppm in methanol extracts of fresh and frozen canola tissues, respectively (Table V). Obviously, an estimate of *s*-triazine concentration based only on methanol extracts would not provide a complete picture of atrazine concentration and metabolism within the tissue.

This study has shown that the described pressure extraction procedure can be used to extract atrazine and metabolites associated with osmotically active water from apoplastic and symplastic compartments of leaves. HPLC analysis of aqueous pressure extracts determined uptake of atrazine, the formation of major metabolites, and accumulation against concentration gradients. GC analysis for metabolites allowed construction of elution patterns and deductions concerning sites of metabolism, form during transport within the plant, and membrane permeabilities. In combination with freeze–thaw treatment, apoplastic and symplastic compartments were identified. It is suggested that with further experimentation the procedure may provide a means to estimate compartment concentrations and therefore may either replace or at least complement existing more time-consuming and limited solvent extraction procedures used in the study of plant herbicide relations.

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Registry No. OH-At, 2163-68-0; de-E OH-At, 19988-24-0; de-P OH-At, 7313-54-4; de-E At, 6190-65-4; de-P At, 1007-28-9; atrazine, 1912-24-9.

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