

The importance of analytical techniques in allelopathy studies with the reported allelochemical catechin as an example

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Abstract Allelopathy can be challenging to demonstrate. Developing rigorous analytical techniques to detect and quantify compound(s) of interest from soil or liquid media lays the foundation for designing ecologically relevant experiments that incorporate candidate allelochemicals. In this paper, fundamental components of analytical techniques, including method development, validation, and appropriate controls are discussed. Research on the candidate allelochemical from spotted knapweed, catechin, is used as an example to demonstrate the importance of including these components both during data collection and in subsequent publications. This example shows how contrasting results between research groups can be difficult to interpret when information on controls and method validation are not included in publications. Recent research suggests that catechin is not likely driving spotted knapweed's invasion, and

this future research on this system should focus on alternate candidate toxins from spotted knapweed. By employing appropriate analytical techniques, such as those outlined here, a strong foundation can be laid for ecologically oriented experiments that examine the role of allelochemicals in structuring communities.

Keywords Allelopathy · Analytical techniques · Catechin · *Centaurea maculosa* · *Centaurea stoebe* subsp. *micranthos* · Invasive species · Novel weapons hypothesis · Spotted knapweed

Introduction

Research on allelopathy has had a tumultuous past, falling in and out of favor with ecologists throughout the past century (reviewed by Willis 1985; Inderjit et al. 2005). Indeed, much of the initial work demonstrating allelopathic interactions between plants was descriptive and lacked rigorous experimentation (Willis 1985). Previously published papers have discussed ways to strengthen the quality of allelopathy research (e.g. Weidenhamer 1996; Inderjit and Weston 2000; Romeo 2000; Inderjit and Weiner 2001), and overall the physiological, chemical, and ecological aspects of this field have improved dramatically (see Thijs et al. (1994); Fomsgard et al. (2006); Macias et al. (2006); and Bertin et al. (2007) for excellent examples of innovative and rigorous research on allelopathy). Allelopathy is currently

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receiving increased attention in invasion biology, as it has the potential to explain the success of some invasive plants (e.g. Ridenour and Callaway 2001; Barney et al. 2005; Cappuccino and Arnason 2006; Dorning and Cipollini 2006; Yang et al. 2007). For example, Prati and Bossdorf (2004) found that the root exudates of the invasive plant garlic mustard inhibited germination of a native North American plant, and such inhibition was ameliorated with neutralization by activated carbon addition. The ‘Novel Weapons Hypothesis,’ which posits that exotic plants dominate ecosystems through allelopathic interference with previously unexposed native competitors (Callaway and Ridenour 2004), links the field of allelopathy with invasion biology. The research leading to the Novel Weapons Hypothesis focused largely on the invasive plants diffuse knapweed (*Centaurea diffusa*) (Callaway and Aschehoug 2000) and spotted knapweed (*C. stoebe* subsp. *micranthos*, often referred to as *C. maculosa*) (Ridenour and Callaway 2001) and its reported allelochemical catechin (Bais et al. 2002, 2003a). It has recently been shown that several of the initial findings regarding the reported allelochemical catechin (e.g. Bais et al. 2003a; Weir et al. 2003, 2004; Perry et al. 2005) are not repeatable (Blair et al. 2005, 2006; Perry et al. 2007), yet the causes of the fundamental discrepancies between studies are difficult to determine due to a lack of published information on method development and the inclusion of positive and negative controls in the original work.

The goal of this paper is to highlight the importance of appropriate and clearly specified analytical techniques in research on previously identified candidate allelochemicals. The ability to detect and accurately quantify candidate allelochemicals in various substrates (i.e. soil) is an important component of research on allelopathy. It is a critical first step that lays the foundation for conducting experiments designed to evaluate the ecological significance of candidate allelochemicals (e.g. Inderjit et al. 2001). This paper addresses why appropriate method development, controls, and publication of the details of the employed methods are necessary components of research utilizing chemical analyses to study allelopathy. The techniques discussed here are basic, and may be more familiar to analytical chemists than to ecologists. After discussing appropriate analytical techniques, research on the reported allelochemical, catechin, is used as an example to illustrate their importance.

Proper analytical methods in allelopathy studies

The first step in an investigation of the allelopathic potential of a plant involves designing experiments that demonstrate allelopathy may contribute to observed negative effects on other plants. Excellent reviews and studies have been published on the challenges of such experiments (Thijs et al. 1994; Weidenhamer 1996; Inderjit et al. 2001; Lau et al. 2008). If a pattern suggesting allelopathy is found, identification of putative allelochemicals through laboratory bioassays should follow (Inderjit and Nilsen 2003). One approach for identifying candidate allelochemicals is to grow the plant in a simplified environment such as sterile liquid media and then analyze the media for toxic root exudates. The process of chemical identification is beyond the scope of this paper, but it should be noted that it requires substantial formal training. Once compounds are identified, sterile culture can also be used to quantify baseline production of putative allelochemicals, as it removes many of the complicating factors encountered when utilizing a complex medium like soil. While these are critical first steps to take, it is important to note that the findings obtained from such studies are not ecologically relevant until further research determines whether the putative allelochemicals are detected in the natural environment and negatively impact ecologically relevant species at realistic concentrations. Inderjit and Weiner (2001) made an excellent point when they stated that more advanced research on allelopathy should be done in the context of soil ecology instead of direct plant-plant chemical interactions. Before such ecologically relevant research can take place with a putative toxin, however, analytical chemistry must be utilized for compound detection and quantification, particularly from natural soil matrices. Analytical techniques thus form the foundation for research on candidate allelochemicals.

Quantifying compounds with, for example, gas chromatography (GC) or high-pressure liquid chromatography (HPLC) from a complex sample matrix such as soil or even relatively simple solutions such as liquid media requires the development of a protocol that efficiently extracts the compound(s) of interest (see Box 1 for a glossary of terminology). For scientists not trained in analytical chemistry techniques, this typically involves searching the literature for previously published methods and/or collaborating with a chemist or

soil biologist. Prior to sample analysis, the recovery efficiency of the technique must be established by spiking the sample matrix with a known amount of the compound(s) of interest (i.e. creating a positive control), extracting the compound(s), and then quantifying the amount of that compound(s) to determine if matrix effects mask or complicate the detection of the compound(s) (Harris 2005, pp. 93–94). Because compounds are often a component of root exudates or mucilage, which may alter compound solubility and/or stability, simply spiking soils provides a much more simplified system than exudation in to natural soils. It is, however, the best approach available to determine recovery efficiency, and it is far worse to not evaluate spiked soil samples. Ideally, recoveries should approach 100% if practically possible, but if not, knowing the percentage recovered from spiked soils can be used in calculating total amounts of compounds present in natural soils. Recovery efficiencies should be determined across a range of concentrations that encompass the range of concentrations from actual samples. Recovery efficiencies are determined by calculating against a standard curve of known concentrations (see Box 1). Often, the existing literature provides a rough estimate of concentrations one might encounter in experiments or in the field, and as preliminary samples are analyzed, the range of the standard curve and positive controls can be refined. Chemical extraction of field soil may yield higher levels of a compound than are actually bioavailable. For example, if a compound is tightly bound to the soil matrix it might not be able to interact with roots, but chemical extraction could remove the compound from the soil matrix. Thus, amounts of a compound found using chemical extraction serve as an upper limit to guide initial tests of the ecological relevance of the compound (i.e. applying the

compound at realistic concentrations to soil in which native species of interest are grown).

It is critical to include the relevant range of positive controls and standards with each set of samples analyzed by the appropriate analytical instrument (e.g. HPLC) to demonstrate that the extraction technique works and that the recovery efficiency does not fluctuate in efficacy through time or with different sample matrices. Blanks (negative controls) must also be included with each set of samples to ensure that other compounds within the sample matrix do not inflate measurements of potentially allelopathic compound(s) (Harris 2005, p. 93). Finally, determining the compound's stability, not only in the sample preparation and analysis phases, but also under environmental conditions, is an important component of method development. For example, it is important to know if a compound is unstable in stock solutions, as standards made from these solution would fluctuate through time, or if the compound is sensitive to environmental conditions, like UV light, pH, or temperature. This is important when collecting and storing field samples for analysis. The experimental principles discussed here are not novel; highly regulated fields such as the pharmaceutical industry consider these standards as fundamental components of 'Good Laboratory Practice' (GLP) (<http://www.epa.gov/Compliance/monitoring/programs/fifra/glp.html>), and these principles are discussed in depth in analytical chemistry texts (see Kellner et al. (2004) and Harris (2005) for more detailed discussions of the topics covered here).

The novel weapons hypothesis, spotted knapweed, and catechin

The Novel Weapons Hypothesis (Callaway and Ridenour 2004) has been suggested as one potential

Box 1 Analytical chemistry terminology used in method development and method validation

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|---------------------------------|---|
| Positive control | Spiking a sample matrix (i.e. liquid media or soil) with known amounts of a compound(s) and then analyzing with a chosen extraction technique the efficiency of a technique. |
| Blanks (i.e. negative controls) | Analyzing the sample matrix devoid of the compound of interest with the chosen extraction technique to ensure that no other compounds within the matrix have similar retention times, which could lead to inaccurate measurements. |
| Standard curve | When quantifying variable amounts of compound(s) in a sample matrix, one must run standards of known concentrations that cover the range of values found in samples to accurately calculate a concentration. Extrapolation of amounts beyond this curve introduces potential error in the quantitation. |
| Recovery efficiency | The percentage of a compound that can be recovered from a sample matrix. This is a critical number to know, as low numbers may result in invalid data. |

explanation for the dramatic success of the invasive *Centaurea* species (especially spotted knapweed) in North America (Bais et al. 2003a; Callaway and Ridenour 2004; Vivanco et al. 2004). Ridenour and Callaway (2001) published compelling data suggesting that a significant amount of spotted knapweed's ability to interfere with the growth of a native North American grass was associated with allelopathy, although at that time putative allelochemicals had not been identified. In 2002, Bais et al. identified a potential allelochemical exuded from spotted knapweed roots ((-)-catechin), which was reported to have 'broad-spectrum herbicidal activity.' Since that initial publication, other studies from the same research group have been published on the role of (\pm)-catechin (catechin is exuded as a racemic mixture of (-)-catechin and (+)-catechin) in spotted knapweed invasion. These studies examined quantitation of catechin production from plants grown in liquid media (Bais et al. 2002; Bais et al. 2003a; Weir et al. 2003, 2004), impact of catechin on plant growth (Bais et al. 2002; 2003a; Weir et al. 2003), mode of action (Bais et al. 2003a), quantitation of catechin in field soil from spotted knapweed-infested sites (Bais et al. 2002, 2003a; Perry et al. 2005, 2007; Thelen et al. 2005), impact of herbivory by biological control agents on catechin exudation (Thelen et al. 2005), biosynthesis of catechin (Bais et al. 2003b), and oxalate secretion by native plants conferring catechin resistance (Weir et al. 2005).

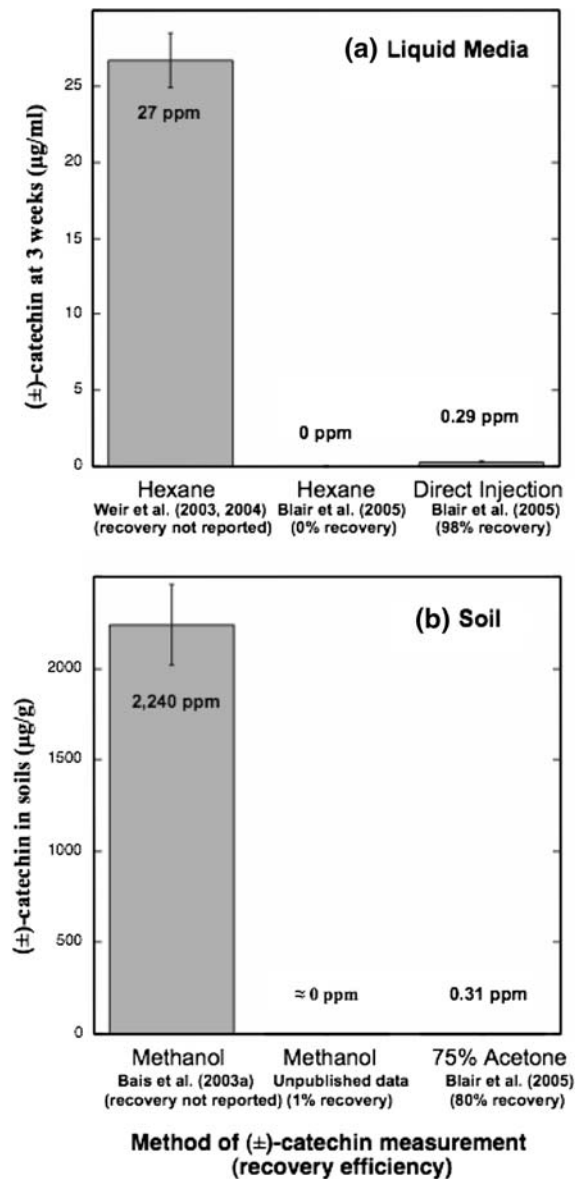
The idea that a single compound could explain the invasion success of spotted knapweed was original and intriguing to many ecologists. Fundamental problems with previously published methodologies for analyzing catechin in liquid media and soil (Bais et al. 2002, 2003a; Weir et al. 2003; Perry et al. 2005; Thelen et al. 2005), however, prevented replication of previous findings, as discussed in Blair et al. (2005, 2006). Below, research on catechin is used to illustrate the necessity of proper analytical methods in order to validate the techniques utilized during data collection and the conclusions in resulting publications.

The importance of appropriate methods in analytical chemistry: an example with the reported allelochemical catechin

Catechin was first studied from plants grown in sterile liquid media. In the original work (Bais et al. 2002,

2003a; Weir et al 2003, 2004), hexane was used to extract catechin from the liquid media, and these findings served, in part, as the foundation for the Novel Weapons Hypothesis. In a later study that tried to repeat this same technique to extract catechin from liquid media, Blair et al. (2005) found that even at liquid media concentrations of 1,000 ppm, no detectable catechin could be extracted using hexane as the extraction solvent. The Merck Index (Tenth Edition, p. 266) states 'catechin is practically insoluble in non-polar solvents (i.e. benzene, chloroform, petroleum ether)'. Hexane is approximately as non-polar as benzene. Despite this, studies using hexane to extract catechin from liquid media (Bais et al. 2002, 2003a; Weir et al. 2003, 2004) report high catechin concentrations (e.g. 27 ppm, Weir et al. 2003, 2004). Interpreting these results is difficult because none of the previously published papers that used the hexane extraction protocol (Bais et al. 2002, 2003a; Weir et al 2003, 2004) reported recovery efficiencies or the inclusion of blanks. Data from a new, 98% efficient direct-injection approach to measure catechin in liquid media (see Blair et al. 2005 for details on method validation and protocol) make the previous data even more difficult to interpret. Using comparable growing conditions to Bais et al. (2002) and Weir et al. (2003), and the direct injection approach, Blair et al. (2005) found two orders of magnitude less catechin than was reported from the original hexane studies (Fig. 1a).

Issues also arose with the analytical method used to quantify catechin in natural soils. Previous studies reported using 100% methanol to extract catechin from soil (Bais et al. 2002; Thelen et al. 2005; Perry et al. 2005). Using catechin spiked soil samples, the methanol extraction technique provided only 0–17% recovery, with the range reflecting the variety of soil types used (Blair et al. 2005). Such low recoveries could lead to inaccurate estimates of the compound in natural soil. Thus, nine solvent systems were then tested before selecting one (75% acetone, 25% water, 0.1% H_3PO_4), which gave adequate recovery efficiencies for a variety of soil types (for dry soils ranging from a low of 16% for one soil to >80% for four other soils) (Blair et al. 2005). The initial studies that used methanol extraction (Bais et al. 2002; Thelen et al. 2005; Perry et al. 2005) did not include recovery efficiencies or report whether blanks were included to check for matrix interferences. While it is possible that these previous studies analyzed soils that had higher recovery efficiencies than the soils



used in the method development by Blair et al. (2005), that is impossible to determine without published data on recovery efficiencies. With the methanol technique, past studies (Bais et al. 2002; Thelen et al. 2005; Perry et al. 2005) reported levels of catechin from soils taken from natural spotted knapweed infestation more than three orders of magnitude higher than Blair et al. (2005, 2006) (Fig. 1b).

A recent study by Perry et al. (2007) sheds some light on the discrepancies among studies. Perry et al. (2007) examined soil catechin levels in 402 samples from 11 spotted knapweed sites throughout a year.

◀ **Fig. 1** Comparisons of reported levels of (±)-catechin. **(a)** From spotted knapweed plants grown in liquid media and analyzed using hexane extraction (Weir et al. 2003, 2004) or direct injection (Blair et al. 2005). Data were extracted from Fig. 1 of Weir et al. (2003, 2004) using ImageJ for the first bar. Data for the second and third bars were taken from values in the text of Blair et al. (2005). See the texts for detailed techniques and HPLC parameters. **(b)** From natural soil samples of spotted knapweed rhizospheres analyzed using methanol (Bais et al. 2003a) or acidified 75% acetone (Blair et al. 2005, 2006). Data were extracted from Fig. 1a of Bais et al. (2003a) using ImageJ for the first bar. The second bar is from unpublished data in which the authors on this paper used methanol extraction on soil from a spotted knapweed site in Montana, USA. Methanol provided only 1% recovery efficiency for that soil based on a standard curve, but Perry et al. (2007) found recovery efficiency of close to 17% from several different soils. The third bar is from Blair et al. (2006) in which an acetone solvent system that gave 80% recovery efficiency was used to assay soils from three spotted knapweed sites in Montana, USA throughout a growing season. Blair et al. (2006) detected trace levels of catechin in 16 out of 45 assayed soil samples, and the average presented in the third bar includes only the samples that contained catechin, and thus is likely an overestimate of catechin presence in knapweed infested soils across the landscape

Using the same methanol extraction technique (e.g. Bais et al. 2002; Perry et al. 2005) and reporting a recovery efficiency of <17%, Perry et al. (2007) detected catechin at only one site on one sampling date, although the catechin concentration was as high as in previous reports (650 ppm, or ≈ 3,800 ppm if one accounts for the 17% recovery efficiency). Additionally, Perry et al. (2007) state that they found high levels of catechin in two sets of blanks; data from such sites were appropriately removed from their study. Because of the contamination, Perry et al. (2007) suggest that previous results not including blanks (e.g. Bais et al. 2002; Thelen et al. 2005; Perry et al. 2005) should be regarded with caution.

In a further complication with this system, catechin was found to have limited stability in aqueous media at room temperature (Blair et al. 2005), supporting previous findings (Ho et al. 1995; Zhu et al. 2002). With this knowledge, steps were taken by Blair et al. (2005, 2006) (i.e. acidifying the sample in this case) to increase sample stability and enhance accurate quantitation of catechin concentrations in liquid media. Likewise, the development of the soil extraction method demonstrated that catechin stability was highly sensitive to and negatively affected by soil moisture and pH (Blair et al. 2005). Catechin was highly unstable in a moist soil matrix above pH 5.5. Because soils in the intermountain west rangelands,

where spotted knapweed is a major invader, are generally well above pH 5.5 and experience periods during the year in which soils are fully moist, this stability information was critical to understanding why the recovery of catechin from natural soils was so low (Blair et al. 2005, 2006). This in turn led Blair et al. (2006) to the final conclusion that catechin is not a likely viable allelopathic agent. Furubayashi et al. (2007) recently corroborated these findings; they found that (+)-catechin was unstable in soils, and this instability resulted from the presence of the catechol moiety. Based on their findings, they calculated that catechin would have to be released at levels at least three times higher than reported in Bais et al. (2003a) to have an allelopathic effect (Furubayashi et al. (2007); recall that Blair et al. (2006) found three orders of magnitude less catechin than reported by Bais et al. (2003a)). Furubayashi et al. (2007) conclude by stating that ‘the phytotoxicity of all catechol-bearing compounds released from plants into soils is likely to be substantially reduced by adsorption and transformation reactions.’ Perry et al. (2007) suggest that in spite of the infrequent presence of catechin in the field, this compound could influence spotted knapweed ecology through, for example, episodic effects on competitors or by producing frequent but transient pulses. Additionally, it is plausible that if spotted knapweed acidifies its rhizosphere, as other plants are known to do (Hinsinger et al. 2003), catechin could be stabilized in that region and perhaps mediate allelopathic interactions between close neighbors with overlapping root systems. Nevertheless, Perry et al. (2007) conclude by stating that ‘the infrequency of soil catechin weakens the hypothesis that it plays a role in *C. maculosa* invasions.’ While catechin may not be driving spotted knapweed invasion, the study by Ridenour and Callaway (2001), which demonstrated that spotted knapweed has the potential to exert allelopathic effects on natives through a greenhouse carbon addition experiment, suggests that it is worthwhile to return to the bioassay step to find alternate candidate toxins.

Concluding remarks

As scientists employ analytical techniques to study allelopathy (including further testing of the Novel Weapons Hypothesis), it is important that awareness

exists regarding appropriate development of analytical chemistry methods and use of appropriate controls. Collaborations between ecologists and analytical chemists and/or soil scientists, coupled with a basic understanding of how analytical techniques are performed, will aid population and community ecologists involved in this line of research. A number of rigorously executed studies serve as excellent examples for those pursuing research on allelopathy. For example, recent experiments with cultivated fine fescue, sorghum species, and wheat have identified putative allelochemicals released from plant root exudates or residues, followed their degradation over time in soil settings, addressed their respective modes of action on higher plant growth, and identified bioactive allelochemicals or their metabolites at levels that could account for significant allelopathic interference in field settings (Nimbal et al. 1996a, b; Bertin et al. 2003; Dayan 2006; Macias et al. 2006; Bertin et al. 2007). Fomsgaard et al. (2006) provide an excellent example of rigorous and well-documented method validation (i.e. reporting the inclusion of blanks, a standard curve, and recovery efficiencies from soil). Clearly, such studies demonstrate that allelopathy remains an important field that warrants further investigation. Inclusion of appropriate analytical techniques in future research on allelopathy and in the resulting publications will strengthen the field and prevent further discrepancies and confusion in the literature.

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