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Using the Copse snail *Arianta arbustorum* (Linnaeus) to Detect Repellent Compounds and the Quality of wood Vinegar

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ABSTRACT: Wood vinegar is the aqueous phase of the liquid produced during the slow pyrolysis of wood. It has the potential to be used as a pesticide against various weeds, insects and molluscs. Due to divergent feedstocks, pyrolysis processes and storing conditions, the chemical composition of wood vinegar varies between producers and time. The aim of our current study was to use the copse snail *Arianta arbustorum* as a biological odour detector to identify the effective compounds behind the repellent effect of wood vinegar. We also studied whether variation in the chemical composition of wood vinegars from different producers impacts repellency efficiency. Of the tested constituents, acetic acid, furfural and ether-soluble (mainly aldehydes, ketones, lignin monomers) and ether-insoluble ("wood syrup") fractions of the water extract of wood vinegar induced a clear repellent effect on snails, but their effects were considerably lower than the effect of wood vinegar. Thus the repellent effect of wood vinegar is due to a larger set of its chemical constituents rather than to a specific compound. All tested wood vinegars induced a clear repellent influence on snails, but differences existed between the products of different retorts. These differences were at least partly due to differences in the products' organic material content. According to our studies, *A. arbustorum* can sense quality differences between wood vinegars, even below 10% dilutions. We suggest that utilizing the avoidance behaviour of *A. arbustorum* is an easy, non-costly method for monitoring the quality of slow pyrolysis liquids but also hitherto unknown environmental contaminants.

Key words: *Arianta arbustorum,* Wood vinegar, Repellent, Pyrolysis liquids, Slow pyrolysis

INTRODUCTION

The increased use of pesticides and their impacts on terrestrial and aquatic environments have become a matter of considerable concern during recent decades. Synthetic pesticides especially are suggested to be replaced by compensatory substances that exert a lower risk to the environment (2009/128/EC). Global climate change is concurrently causing alterations in temperature and rainfall patterns, resulting in pest ranges expanding to higher latitudes (Parmesan, 2006; Rosenzweight *et al*., 2001). Consequently, new methods for controlling the damages caused by various pests are needed. Plant-derived products may have a significant role in sustainable plant protection when functioning as compensatory substances for synthetic pesticides. Wood vinegar, also known as pyroligneous acid and wood distillate, is an aqueous phase of the liquid produced during the slow pyrolysis of wood materials. In Asia, wood vinegar has traditionally been used as a biocide against e.g. micro-organisms (Baimark and Niamsa, 2009; Velmurugan *et al*., 2009), weeds and insects (Yatagai *et al*. 2002). The potential of wood vinegar as a pesticide has also been examined in Finland, with promising results (Lindqvist *et al*., 2010; Tiilikkala and Segerstedt, 2009) and negligible environmental risks (Hagner *et al*., 2010a, Hagner *et al*., 2010b,; Hagner 2013). During the last decade, the use of wood vinegar derived from various plant materials has rapidly increased, and numerous botanical pesticides have entered the market especially in several Asian countries, but not in Europe (Tiilikkala *et al*., 2010). The inclusion of wood vinegar as a plant protection product in European Union (EU) markets requires approval and registration as an active substance according to the Plant Protection Products Regulation (1107/2009) issued by the EU. However,

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when applied as a biocide the approval must meet the requirements set by the Biocidal Products Directive 98/8/EC (BPD) (Hagner, 2013). When a chemical mixture is an outcome of a particular process, such as slow pyrolysis, and/or from various sets of parent materials, it is unclear which compounds act as active substances and which are non-acting impurities. It is also likely that such mixtures do not have a well-defined chemical composition (IGHRC, 2009), which further complicates the registration of botanicals as pesticides. The chemical composition of wood vinegar consequently varies between producers and time due to the divergent feedstock, pyrolysis process, storing time and conditions (Fagernäs *et al*., 2012b; Oasmaa *et al*., 2010). To our knowledge the effect of this variation on the functioning of wood vinegar as a pesticide has not been studied. Despite the long history of applying wood vinegar in Asia (Ogawa and Ogimori, 2010), information on the active compounds causing the pesticidal effect of wood vinegar is also lacking. To facilitate the commercialization and product development of wood vinegar as a pesticide in Europe, it is essential to examine the variation in wood vinegar quality and uncover the active compounds behind the pesticide effects. Steering molluscs by means of olfactory cues is a well-known phenomenon (Inoue *et al*., 2004; Kirino *et al*., 2005). Repellent odours induce mantle shortening and behavioural withdrawal from the odorant (Inoue *et al*., 2004; Voss, 2000). Some plant extracts, such as from *Allium sativum*, *Saponaria officinalis* and *Valerianella locusta*, are known to have a repellent effect on the behaviour of slugs (Barone and Frank, 1999; Inoue *et al*., 2004). In our earlier studies we found that wood vinegar/tar made from birch (*Betula sp.*) also has the potential to be used as a snail and slug repellent (Lindqvist *et al*., 2010). Interestingly, a compound or compound group in birch-derived wood vinegar appears to act as an efficient repellent against both slugs and snails. When confronted with wood vinegar, the molluscs stop at a distance of approximately 1 cm from the substrate, and turn around to escape the obviously unpleasant odour (Lindqvist *et al*., 2010). The aim of our current study was to use *A. arbustorum* as a biological odour detector to identify the effective compounds behind the repellent effect of wood vinegar. We also studied whether potential variation in the chemical composition of wood vinegars from different producers impacts repellency efficiency. Further, the effect of wood vinegar ageing on its repellency was examined.

Our study is part of a larger research programme investigating the compositional and chemical characteristic variations and the application targets of wood vinegar (Fagernäs *et al*., 2011a, Fagernäs *et al*., 2011b).

MATERIALS & METHODS

The wood vinegars (Wv) used were the aqueous phases separated from the liquid products of three different slow pyrolysis retorts in Finland (Fagernäs *et al*., 2012b). The retorts are denoted here as A, B and C, and their wood vinegar products as WvA, WvB and WvC, respectively. Retorts A and B are batch retorts (one carbonization oven, carbonization time 24 h, 450°C) and retort C is a continuous retort (carbonization took place in several ovens, carbonization time 2.5 h, 450°C). The feedstocks used in the pyrolysis were unbarked small wood materials or bark-free birch (*Betula pendula)* material from a plywood mill. The complete pyrolysis process and the composition of wood vinegars were analyzed at the VTT Technical Research Centre of Finland (for a detailed description of the methods, see Fagernäs *et al*., 2012a and Fagernäs *et al*., 2012b). The chemical properties of WvA and WvB were quite similar, while those of WvC differed from the others with a much higher proportion of organic matter (Fagernäs *et al*., 2012 b). WvA and WvB contained no settled tar. Instead, the amount of soluble tar (obtained by the addition of water) in WvC was 7%. The main characteristics of wood vinegars are presented in Table 1.

The snails (*A. arbustorum*, Linnaeus) were collected from a grove in the city of Lahti, Southern Finland. Only adult snails were used. Maturity of the adult snails was ensured by identifying the thickened outer lip framing the aperture of the shell (Raboud, 1986). The snails were collected on the morning of the testing and placed in a refrigerator (+4°C) prior to testing. Two hours before the tests the snails were

Table 1. Characteristics of the tested wood vinegars (Fagernäs *et al*., 2012 b)

	WvA	WvB	WvC
pH	2.0	1.8	3.1
Organic matter %	25.3	27.5	57.0
PAH mg/kg	21	120	290
TOC g/L	130	130	290
Acetic acid g/kg	100	130	130
Methanol g/kg	15	18	5.5

Table 2. Model solution concentrations.

Compound	g/L
Furfural	6.9
Acetic acid	110
Guaiacol	1.1
Methanol	17
Syringol	0.1
1-OH-2-propanone	0.17

awakened by spraying them with warm water (20–25°C). The individuals woken within one minute were selected and immediately used in the experiments.

The repellent effect of wood vinegars from various retorts was tested using *A. arbustorum*. The tests were conducted in a fume hood in the laboratory of the University of Helsinki, Finland. Test arenas were constructed out of circular plastic rings (outer –" 12 cm, inner –" 6 cm) with the 3-cm wide plastic area painted with wood vinegars. As wood vinegar forms drops when spread on plastic rings, the wood vinegars (WvA, WvB, WvC) were mixed with Vaseline® to ensure an even spread on the plastic test arenas. The rings were placed on burlap fabric moistened with tap water (to keep the snails active), and the snails were positioned in the middle of the plastic rings on the burlap. The plastic rings were treated either with 1) the mixture of wood vinegar (either WvA, WvB or WvC) and Vaseline® (v/v 1:9), 2) Vaseline® only or 3) no additions (=control), each treatment having seven replicates. The amount of each mixture was 1 g /sheet. The distance between the test units (plastic rings) was 10 cm. Leaves of *Taraxacum officinale* were placed outside the rings to lure the snails into leaving the rings. Three snails were placed inside each ring. The snails' progress was monitored 15 min, 30 min, 1, 2, 3, 4 and 5 h after being placed inside the rings. Snails that left the rings were removed from the experimental area. The experiment was conducted one and six months after the manufacture of the wood vinegar, to assess the effect of ageing on the repellent effect. The wood vinegars were stored at room temperature (20°C) between the tests.

The compositions of the wood vinegars were analyzed at VTT (Fagernäs *et al*., 2012a, Fagernäs *et al*., 2012b). Four of the detected major components (acetic acid, furfural, methanol and 1-hydroxy-2-propanone) and two lignin monomers (guaiacol and syringol) typical of deciduous trees were chosen for further studies to investigate potential constituents causing repellency. The model solutions were prepared at VTT. Compound concentrations in the model solutions were set as equivalent to those in typical wood vinegar (Fagernäs *et al*., 2012b) (Table 2). The liquids were diluted with distilled water to show the differences in the repellent effect of various compounds (v/v 1:2 vs. 33% dilution). Our pre-examinations showed that differences between compounds are easier to detect when using diluted samples. Circular cardboard rings (outer –" 12 cm, inner –" 6 cm) with a 3-cm wide cardboard area were placed in Petri dishes filled with the model solution (v/v 1:2 H_2O), and left to saturate for five minutes (n=7). The rings were then immediately placed on moist burlap fabric. As with the previous test, leaves of *T. officinale* were placed outside the rings. Three snails were placed inside each ring. The snails progress was monitored 2, 5, 10, 15, 20, 30, 45 and 60 minutes after being placed inside the rings. Snails that left the rings were removed from the experimental area. The repellency effect of furfural and acetic acid was also tested using more diluted samples (1:7 H_2O ~15% dilution), a mixture of the two compounds and water (1:1:5 furfural:acetic acid:H_2O). Results were compared to the repellent effect of water (control, no repellent effect) and WvA, which was taken to represent a typical Finnish hard wood vinegar.

The repellent effect of wood vinegar without acetic acid and other volatile compounds was tested using a solvent extraction scheme based on water extraction (Fagernäs *et al*., 2012 b, Sipilä *et al*., 1998). According to this method, wood vinegar WvA (10 g) was dissolved in water and the water-soluble fraction was extracted using diethyl ether. The ether soluble-fraction (ether-solubles) mainly contains aldehydes, ketones and lignin monomers, and the ether-insoluble fraction (ether-insolubles) consists of sugar-like materials (mainly "wood syrup"). As the volumes of evaporation residues were small (4.8% and 4.4% for ether-solubles and ether-insolubles, respectively), the residues were diluted in distilled water (10 ml) to obtain compound concentrations corresponding to that of wood vinegar. Acetone was used to transform evaporation residues into liquid form. After dilution, acetone was evaporated using nitrogen vaporizer. The repellent effect of evaporation residues was tested separately by placing a divided filter paper on a Petri dish (9-cm diam.). A 2-mm space was left between the filter paper halves. One half was saturated with distilled water (0.5 ml) and the other with an evaporation residue (ether or sugar, 0.5 ml, n=4). The homogeneous distribution of snails was controlled in the Petri dishes, in which both sides were treated with water. Three snails were placed in the middle of a Petri dish, which was then covered with a lid. The location of the snails on the i) control paper, ii) paper with evaporation residue or iii) lid was observed after 15, 30, 60, 120, 180 minutes and 24 hours. To test differences in the repellent effect between (i) the various wood vinegar products and (ii) the separate components of the wood vinegar (acetic acid, furfural, methanol, 1-hydroxy-2-propanone, guaiacol and syringol) on snails, a repeated measure ANOVA was conducted. Paired comparisons were made using the Tukey Post Hoc test. Results were compared to the repellent effect of the control sheets (treated with water). The effects of neither ether-solubles nor ether-insolubles were tested statistically due to the interdependence between the control and vaporization product treatments

RESULTS & DISCUSSION

Due to divergent feedstocks and pyrolysis processes the chemical composition of wood vinegars is known to vary between producers (Fagernäs *et al.*, 2012b; Oasmaa *et al.*, 2010). We examined whether birch wood vinegars produced by three different retorts (A, B, C) differ in their repellency for the snails. *A. arbustorum* proved to be a suitable test species as it sensed the differences in wood vinegar compositions even below 10% concentrations. Each wood vinegar product (WvA, WvB, WvC) induced a clear influence on snails when compared to the controls ($p<0.001$ in all cases). Vaseline® also appeared to repel the snails to some extent, but the effect was not statistically significant ($p=0.09$) (Fig.1).

Although each of the tested wood vinegars induced a clear repellent influence on the snails, differences existed between products of different retorts: wood vinegar derived from the continuous retort C (WvC) was most effective, while WvA and WvB did not differ significantly from each other (Fig. 1). This may be due to the high proportion of organic matter (57% in WvC versus ~26% in WvA and WvB; Fagernäs *et al.*, 2012b) and some polycyclic aromatic hydrocarbon (PAH) compounds in WvC (Table 1). The main difference in the organic material of the distillates was the wood syrup (ether-insolubles) content, which was 23% in the organic material of WvC and 2% to 5% in WvA and WvB respectively (Fagernäs *et al.*, 2012b). The repelling effect of the ether-solubles (mainly aldehydes and lignin monomers) and ether-insolubles (“wood syrup”) compound groups as evaporation residues was clear: during the first hour of the test none of the snails remained on the filter paper saturated with ether-solubles (Fig. 2). The repellent effect of ether-insolubles was also clear, with only few snails remaining on the filter papers. Over 50% of the snails escaped to the lids, and these individuals were removed from the analyses. The snails positioned themselves evenly (50% ± 10%) on both halves of the Petri dishes when both halves had been treated with distilled water. The position and behaviour of the snails was observed again after 24 h. In the Petri dishes containing ether-solubles and ether-insolubles 9 snails out of 12 and 6 snails out of 12, respectively, were inactive and had excreted a slime plug in the frontal aperture of their shells. Contrastingly, only one snail out of 12 was inactive in the control Petri dishes with filter papers containing distilled water. The amount of excrements was concurrently observed in the Petri dishes: ample amounts of faeces were detected on the lids and control filter papers, while filter papers containing ether-solubles and -insolubles were clean. However, the proportions of acetic acid and furfural, which were also found to repel snails (see below), did not differ between the three retort products (Fagernäs *et al.*, 2012b). To conclude, differences in the repellent effect of birch-derived wood vinegars from different producers were at least partly due to the increased organic material and thus different water content of the products.

The potential of birch wood vinegar as a pesticide has been proven earlier (Velmurugan *et al.*, 2009; Yatagai *et al.*, 2002; Lindqvist *et al.*, 2010; Tiilikkala and Segerstedt, 2009), but information on the active compounds causing the pesticidal effect of wood vinegar is lacking. In our current study the objective was to shed light on the specific wood vinegar

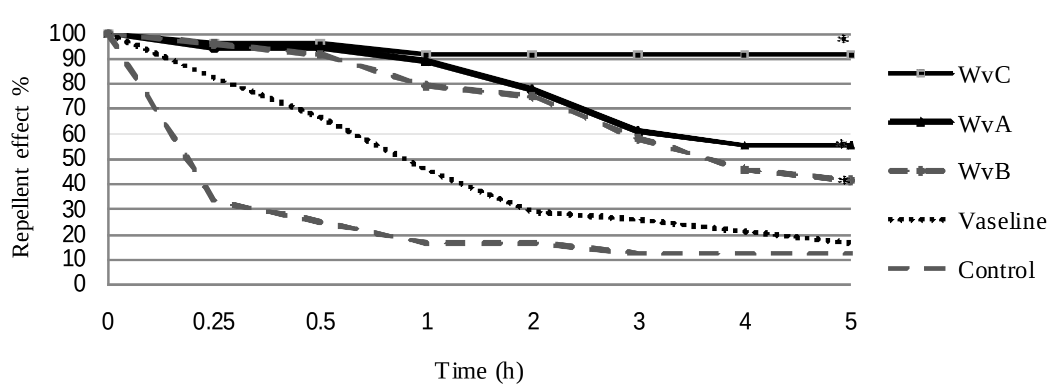

Fig. 1. Repellent effect of the three wood vinegars (WvA, WvB, WvC; 10 % mixture) and Vaseline® on snails one month after manufacturing in relation to control (n=7).

Treatments with asterisk denote a significant difference in relation to control (Tukey, $p<0.001$ in all cases).

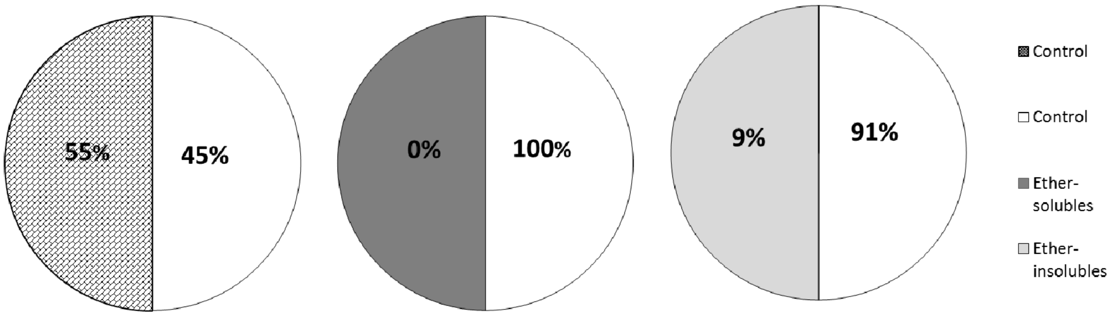

Fig. 2. Location of snails (%) in the two halves (with or without test liquid) of the Petri dishes at 180 min after being placed on the Petri dishes (averages).

The homogeneous distribution of snails was controlled with the control Petri dishes (left), in which both sides were treated with water. In the actual test dishes one half of each dish was saturated with distilled water and the other half with either ether-solubles (middle) or ether-insolubles (right) (n=4). Snails found on the lid were excluded from the graph.

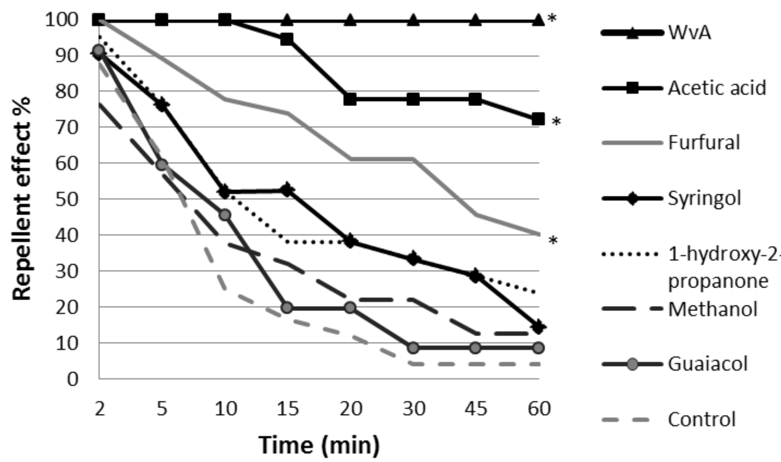

Fig. 3. Repellent effect of wood vinegar (WvA) on snails in relation to its main compounds (33% dilutions).

Treatments with asterisk denote a significant difference in relation to control (Tukey, p=0.00, p=0.01, respectively), n=7

compounds causing repellent effects on snails. Of the tested substances, acetic acid and furfural (33%) had a statistically significant repellent effect on snails (p=0.00 and p=0.01, respectively) (Fig. 3). Syringol and 1-hydroxy-2-propanone also seemed to repel snails, but the effect was not statistically significant (p=0.64, p=0.69). The repellent effect of wood vinegar (WvA) alone was higher than any of its constituents (Fig. 3). When acetic acid and furfural were diluted to 15% (1:7 H_2O), their repellence effect diminished and was not statistically significant (p=0.26, p=0.70). Instead, the mixture of acetic acid and furfural (1:1:5 furfural:acetic acid:distilled water) still induced a clear repellent effect on snails (p=0.01). However, this effect was remarkably weaker than the repellent effect of wood vinegar (WvA) (Fig. 4). These results were expected as the acetic acid and furfural have long been applied in plant protection (Abouziena *et al*. 2009; Hensley and Burger, 2006). Acetic acid is an approved pesticide in Europe and listed as a biopesticide in the United States of America (USA). Furfural has been used due to its antifungal and nematicidal activity. Although acetic acid and furfural exerted a significant repellent effect on snails, their effect, either alone or combined was considerably lower than that of wood vinegar. The repellent effect of the compound groups: ether-solubles (aldehydes, ketones and lignin monomers) and ether-insolubles ("wood syrup") of wood vinegar without acetic acid, was also clear. These observations indicate that the repellent effect of wood vinegar is due to a larger set of its chemical constituents rather than due to a specific compound. The 6-month storage at room temperature did not lower the repellent effect of the wood vinegars. This is in line with Fagernäs *et al.* (2012b), who detected no obvious chemical changes in the aqueous phase of wood vinegar during 6-month storage at room temperature. The amount of organic compounds (mainly acetic acid and furfural) decreased ca. 7% during storage (Fagernäs *et al*., 2012b), but this did not appear to affect the repellent efficiency of wood

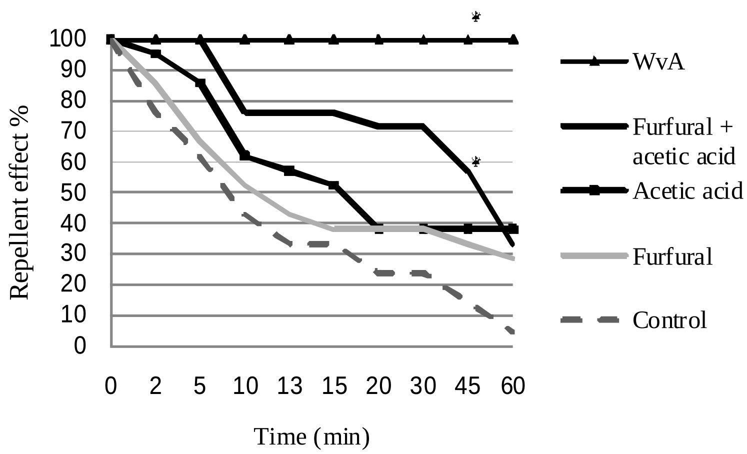

Fig. 4. Repellent effect of furfural, acetic acid and their mixture in comparison to WvA and control (15% dilutions, n=7).
Treatments with asterisk denote a significant difference in relation to control (Tukey p=0.00, p=0.01, respectively), n=4.

vinegar against snails in our current study. This indicates that the repellent effect of wood vinegar may work at even lower concentrations than those applied in our current study (10%). The commercialization of biopesticides depends very much on product duration because very often biobased control agents do not last long during storage or transportation. It is noteworthy that the common pine tar, an equivalent product to that derived from birch wood, is much less effective at repelling molluscs (Hagner, 2005). We assumed that this is due to differences in the lignin monomers of hardwood and softwood: pine wood contains mainly guaiacyl-lignin, while hardwoods contain both guaiacyl- and syringyl-lignin monomers (Sjöström, 1993). However, syringol and guaiacol had no repellent effect on snails in our study (Fig. 3) and thus differences in the repellent effect of pine- and birch-originated liquids were not explained by differences in lignin monomers. It must be mentioned that we used 33% dilutions in our studies and the effect of stronger solutions cannot be discounted. However, this surprising difference is likely due to a lower amount of repelling volatile compounds in pine tar.

Orihashi *et al*. (2001) studied the deterrent effect of wood tar against the gray-sided vole (*Clethrionomys rufocanus bedfordiae*) and found that wood vinegar (without the less-soluble tar fraction) did not reduce vole barking. The authors concluded that tar fraction is crucial in deterring voles. However, in our present study wood vinegar and its separated fractions excluding tar components efficiently repelled snails (see also Lindqvist *et al*., 2010). The increased repellent effect of WvC could also partly be due to its greater PAH concentration, although WvA and WvB with low PAH contents also effectively repel snails. This observation is important as toxic PAH compounds are usually condensed in the tar fraction of the slow pyrolysis products (Fagernäs *et al*., 2012a). Registration and commercialization of botanicals with high PAH content would be difficult. In addition to snails and slugs, recent studies have demonstrated that wood vinegar also repels other species, e.g. psyllids (*Trioza apicalis*) and termites (*Reticulitermes speratus* and *Coptotermes formosanus*) (Oramahi and Yoshimura, 2013; Tiilikkala and Segerstedt, 2009), while the beetle *Meligethes aenneus* was not repelled by wood vinegar (Tiilikkala and Segerstedt, 2009). These observations indicate that the repellent effect of wood vinegar varies between species. It is also noteworthy that the effects are expected to be highly variable, as wood vinegars are produced from various types of parent materials.

CONCLUSIONS

The type of feedstock and the varying processes used in slow pyrolysis determine the chemical characteristics of slow pyrolysis liquids. Consequently, the quality control, in regards to testing the repellency effect, of wood vinegars by chemical techniques is challenging due to the variety of potential volatile compounds in the substrate. According to our studies, *A. arbustorum* can sense quality differences of wood vinegars even below 10% dilutions. We suggest that using the avoidance behaviour of *A. arbustorum* is an easy, non-costly method for monitoring the quality of slow pyrolysis liquids. At present the responses of biological organisms, such as fish, algae and microorganisms are widely used to monitor e.g. the quality changes in wastewaters (US"EPA, 2002). Steering molluscs by means of olfactory cues is a well-known phenomenon (Makino and Yano, 2010), but to our knowledge this is not a common practice in industrial applications. Twitching and quivering of the tentacles during snail olfactory orientation is dependent on odour concentrations in the air (Lemaire and Chase, 1998; Nikitin *et al*., 2006). Consequently, in addition to avoidance behaviour, the analysis of snail tentacle movements could be a feasible way to observe even slight quality differences in slow pyrolysis liquids. The use of snails as volatile organic compound detectors is a new innovation and **-** due to the increasing chemicalization of the world **-** the quality control of pesticides and other environmental contaminants is needed.

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