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Determination of free and glucosidically-bound volatiles in plants. Two case studies: *L*-menthol in peppermint (*Mentha x piperita* L.) and eugenol in clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry)

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ABSTRACT

This study arises from both the today's trend towards exploiting plant resources exhaustively, and the wide quantitative discrepancy between the amounts of commercially-valuable markers in aromatic plants and those recovered from the related essential oil. The study addresses the determination of both the qualitative composition and the exhaustive distribution of free and glucosidically-bound *L*-menthol in peppermint aerial parts (*Mentha x piperita* L., Lamiaceae) and of eugenol in dried cloves (*Syzygium aromaticum* (L.) Merr. & L.M.Perry, Myrtaceae), two plants known to provide widely ranging essential oil yields. The two markers were investigated in essential oils and residual hydrodistillation waters, before and after enzymatic hydrolysis. Their amounts were related to those in the headspace taken as reference.

The results showed that the difference between marker compound in headspace and in essential oil amounted to 22.8% for *L*-menthol in peppermint, and 16.5% for eugenol in cloves. The aglycones solubilised in the residual hydrodistillation waters were 7.2% of the headspace reference amount for *L*-menthol, and 13.3% for eugenol, respectively representing 9.3% and 15.9% of their amounts in the essential oil. The amount of *L*-menthol from its glucoside in residual hydrodistillation waters was 20.6% of that in the related essential oil, while eugenol from its glucoside accounted for 7.7% of the amount in clove essential oil. The yield of *L*-menthol, after submitting the plant material to enzymatic hydrolysis before hydrodistillation, increased by 23.1%, and for eugenol the increase was 8.1%, compared to the amount in the respective conventional essential oils.

This study also aimed to evaluate the reliability of recently-introduced techniques that are little applied, if at all, in this field. The simultaneous use of high-concentration-capacity sample preparation techniques (SBSE, and HS-SPME and in-solution SPME) to run quali-quantitative analysis without sample manipulation, and direct LC–MS glucoside analysis, provided cross-validation of the results.

1. Introduction

Volatile compounds are often present in plants as glycosidically-bound components. These compounds were first described by Bourquelot and Bridel (1913) who identified a geranyl-β-D-glucoside in *Pelargonium odoratissimum*. To date, glycosidically-bound volatiles have been found to occur in almost 170 species belonging to 50 families (Crouzet and Chassagne, 1999; Stahl-Biskup et al. 1993; Winterhalter and Skouroumounis, 1997) not only in the aerial parts but also in roots, rhizomes, petals, fruits and seeds. Most of them are O-glycosides whose aglycone moieties belong to different classes of metabolites (mainly phenols and terpenoids). Conversely, the commonest glycone in nature is β-D-glucose, directly bound to the aglycone. The glucose moiety may also be replaced by one or more additional sugar units (Winterhalter and Skouroumounis, 1997). The large number of β-D-glucose derivatives, and the roles they play in plants, were the background for the development of a rapid and effective method, based on enzymatic hydrolysis by β-glucosidase, to determine the content of these compounds in several matrices of vegetable origin, including wines, fruits, and other

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List of acronyms

HD	conventional hydrodistillation
CEO	clove essential oil
CEO-R-EH	clove EO from the distillation of the waters residual after enzymatic hydrolysis
CR	clove residual distillation water
EH-EO	essential oil obtained after plant enzymatic hydrolysis
EO	essential oil
HS	headspace
HS-SPME	headspace solid phase microextraction
Micro-HD	micro-hydrodistillation
MHS-SPME	multiple headspace solid phase micro-extraction
PEO	peppermint essential oil
PEO-R-EH	peppermint EO from the distillation of the waters residual after enzymatic hydrolysis
PR	peppermint residual distillation water
R(s)	residual hydrodistillation water(s)
SBSE	stir bar sorptive extraction

substrates (Winterhalter and Skouroumounis, 1997; Ananthakumar et al., 2006).

In plant tissue, glycosylation is a common protective mechanism that prevents lipophilic volatile compounds (mainly phenols or alcohols) from damaging labile cellular components, such as cell membranes, improves the storage of volatile metabolites within the plant vacuoles, and protects plants from any form of toxicity due to the aglycone (Stahl-Biskup et al., 1993). Moreover, glycoconjugates are considered to be important for accumulation and storage, as well as providing transportable forms of some hydrophobic substances involved in several processes of plant metabolism, and, in particular, as intermediates in the formation of secondary metabolites (Hosel, 1981). As a consequence, it is quite common to find the glycosides of phenolic or monoterpenic alcohols alongside the main components of the essential oil of a plant (Stahl-Biskup et al., 1993). This tendency however cannot be generalised because glycoside-released-aglycones may or may not be present in the corresponding essential oil, as shown by Den Van Dries and Baerheim Svendsen (1989) in a study on free and glucosidically-bound volatiles in fresh needles of *Juniperus chinensis* var. *pfitzeriana* and *J. communis*, in leaves of *Cupres-socyparis leylandii*, and in fresh aerial parts of *Mentha piperita citrata* and *Salvia officinalis*.

Glycoconjugates are generally water soluble, less reactive, non-volatile and odourless compounds, and are considered as potential natural sources of odorous volatile aglycones, releasable by enzymatic or chemical hydrolysis (Nirmala Menon and Narayanan, 1992) during maturation, industrial pretreatment or processing (Stahl-Biskup et al., 1993).

The study of the free and glycosidically-bound volatile composition is useful for a correct evaluation both of the total contents of odorous components that are potentially available from an aromatic plant, and of their total recovery, to be added to that conventionally obtained with essential oil isolation, not least in view of their use in the food and cosmetic fields. Moreover, residual distillation waters after essential oil isolation have also recently been tested to evaluate their effect on growth, productivity, and essential oil content and composition of peppermint (*Mentha* × *piperita* L. 'Black Mitcham') plants, with positive results. These treatments showed significant effects on plant height and weight, essential oil content and yield, as well as essential oil composition (Zheljazkova and Astatkieb, 2012).

In spite of this interest, relatively few publications have dealt with the chemistry of glycosidically-bound volatiles and their distribution in plants. Most such studies have concerned quantitative analysis of hydrolytically-liberated aglycones (Ananthakumar et al., 2006).

During a study aimed at developing a rapid but reliable method for quality control of spices, a marked inconsistency between menthol and eugenol contents in peppermint aerial parts and in cloves, when quantitated in essential oils and via headspace solid phase microextraction (HS-SPME) was observed (Sgorbini et al., 2015).

The aim of this study was therefore (i) to investigate exhaustively the volatile composition of the aerial parts of peppermint (*Mentha x piperita* L., Lamiaceae) and dried cloves (*Syzygium aromaticum* (L.) Merr. & L.M.Perry, Myrtaceae) (ii) to evaluate the distribution of eugenol and *L*-menthol in free form, in the essential oils and in the water phase resulting from hydrodistillation (residual distillation water – R) and to compare these distributions to the "nominal" total amounts in the original matrices, measured by multiple headspace extraction-SPME-GC-FID/MS, and (iii) to analyse the two compounds in their glucosidically-bound forms. *L*-menthol and eugenol were selected as model compounds because (a) they are the most abundant markers of the plants investigated, (b) they may be taken as representative of monoterpene alcohols and phenolic compounds, i.e. two of the groups of secondary metabolites often present in glycoside form in several species, (c) they may be taken as representative of compounds with different water solubility, their octanol/water partition coefficient, $K_{o/w}$, being 3.38 for *L*-menthol and 2.73 for eugenol (Episuite, 2012), and (d) the two plant matrices are known to provide widely differing essential oil yields.

2. Results and discussion

This study comprised two main parts: the first part focused on *L*-menthol and eugenol in their free forms, and the second on their glucosidically-bound forms; each part consists of a number of steps that are summarised in Fig. 1, in particular for *L*-menthol and eugenol free forms:

(1) Headspace analysis of dried plant material by MHS-SPME-GC–MS (HS-1) and quantitation of *L*-menthol and eugenol. Headspace sampling with solid phase microextraction (HS-SPME) makes it possible to evaluate the qualitative composition of the volatile fraction of peppermint aerial parts and cloves, and to quantify *L*-menthol and eugenol present in free form in the original dried plant material (Arthur and Pawliszyn 1990). Quantitation was achieved by the multiple headspace approach (MHS-SPME) (Ezquerro et al., 2003; Bicchi et al., 2011) in consideration of its effectiveness with solid matrices, since it is not influenced by the matrix effect (Kolb and Ettre, 1997). HSSE was also tested (Bicchi et al., 2000; Tienpont et al., 2000) and gave comparable results to HS-SPME; HS-SPME was chosen as being easier to be run automatically. Theory and use of MHS-SPME is reported in paragraph 1SM in the supplementary material.

(2) Hydrodistillation of plant material (EO-1) and determination of normalised % composition, and true quantitation of *L*-menthol and eugenol in the resulting EOs by GC-FID and

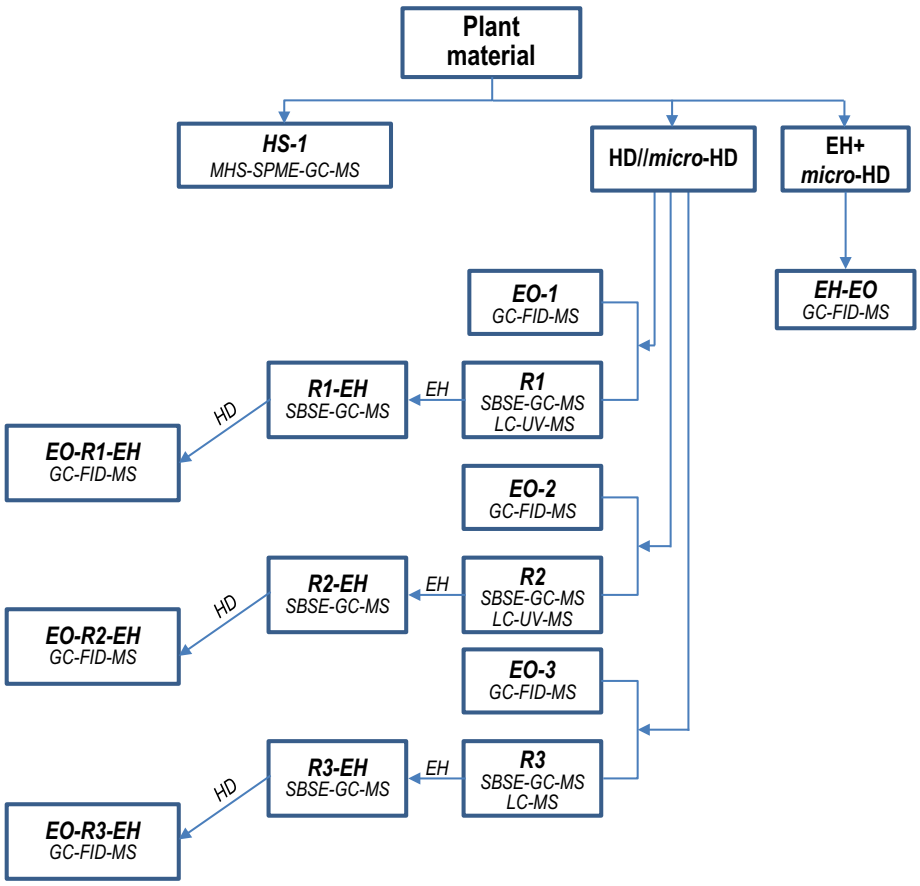

Fig. 1. Scheme of free and glycosidically-bound volatile compounds analysis. EO: essential oil; HS: Headspace; HD: hydrodistillation; EH: enzymatic hydrolysis; EO-EH: EO hydrodistilled after enzymatic hydrolysis of water-soluble glucosides; R1, (2 or 3): Residual distillation water 1 (2 or 3); R1- (2 or 3)-EH: Residual distillation water 1 (2 or 3) submitted to enzymatic hydrolysis; EO-R1 (2 or 3)-EH: EO from R1 (or 2) after enzymatic hydrolysis.

GC–MS. After the first hydrodistillation, both samples were submitted to repeated hydrodistillation, by re-suspending plant materials in "new" freshly distilled water, to isolate EOs exhaustively (EO-2 and EO-3). Residual hydrodistillation waters (R1, R2 and R3) were also stored for further analysis.

(3) All residual waters from the hydrodistillations (R1, R2, R3) were submitted to enzymatic hydrolysis of solubilised glucosides, and again hydrodistilled, and the resulting EOs (EO-R1-EH, EO-R2-EH, EO-R3-EH) analysed by GC-FID and GC–MS to determine normalised % composition, and to quantify *L*-menthol and eugenol. These results are related to the previous steps, because they also provide an indicative recovery of volatiles deriving from hydrolysis of glucosides solubilised in each R.

(4) Quantitation of free *L*-menthol and eugenol in residual distillation waters (R1, R2 and R3) was run by Stir Bar Sorptive Extraction (SBSE) in combination with GC-FID and GC–MS (Baltussen, 1999).

(5) Micro-hydrodistillation (micro-HD) of plant material after maceration and enzymatic hydrolysis (EH) of water solubilised glucosides (EH-EO) and determination of normalised % composition, and quantitation of *L*-menthol and eugenol in the resulting EOs by GC-FID and GC–MS. These analyses aimed to compare yield and % composition of the marker compounds in the samples obtained from the plant material, as such (EO-1) and after maceration/hydrolysis (EH-EO); they also sought to quantify total amounts in free and glucosidically-bound forms and, indirectly, amounts of the related glucosides, and to evaluate the free/glucosidically-bound aglycone ratios using the procedures described here.

The second part, concerning glucosidically-bound *L*-menthol and eugenol, involved:

(6) Quantitation of total *L*-menthol and eugenol (i.e. free + glucosidically-bound) in residual distillation waters after enzymatic hydrolysis (R1-EH, R2-EH, R3-EH) of glucosides that was run by SBSE-GC–MS and GC-FID. Acidic hydrolysis was abandoned because in the preliminary tests carried out on octyl-β-glucoside taken as model compound, significant differences in the 1-octanol abundance depending on the pH of hydrolysis were measured, and extensive formation of artefacts when applied to the first peppermint residual distillation water (PR1) were detected, in agreement with Williams et al. (1982).

(7) Quali- and quantitative results of glucoside analyses in Rs were confirmed by analysing them directly by LC–MS. Eugenyl glucoside was synthesised as reported in paragraph 4.5, while a 78% pure *L*-menthol glucoside sample was available from the collection of standards in the authors' laboratory.

The results of the set of analyses of each plant matrix are, for clarity, collected in two separate paragraphs (2.1 for peppermint aerial parts and 2.2 for clove). *L*-Menthol and eugenol were taken as representative markers of the investigated plant materials; the quantitative results of this study are all expressed as ppm or mg of *L*-menthol and eugenol, i.e. as absolute amounts in 100 g of plant material or in 2 l of residual distillation waters; they are summarised in Table 1a–c. Concentration range, linear regression equations, and determination coefficients for *L*-menthol and eugenol

Table 1
Free and glucosidically-bound *L*-menthol and eugenol quantitative results obtained by GC–MS or GC-FID and LC–MS in the various steps of the present study: (a) free *L*-menthol and eugenol quantitative data in HS, EO and Rs expressed as mg in 100 g of plant material; (b) glucosidically-bound *L*-menthol and eugenol quantitative data, after enzymatic hydrolysis and as such, expressed as ppm and mg in 100 g of plant material; (c) summarising quantitative data. C: clove; P: peppermint aerial parts; EO: essential oil; HS-1: Headspace of plant material; EH: enzymatic hydrolysis; EO-1: exhaustively isolated EO; EH-EO: EO isolated after enzymatic hydrolysis of water-soluble glucosides; R1 (2 or 3): Residual distillation water 1 (2 or 3); R1- (2 or 3)-EH: Residual distillation water 1 (2 or 3) after enzymatic hydrolysis; EO-R1 (or 2)-EH: EO from R1 (or 2) after enzymatic hydrolysis; Rs: Residual distillation waters.

		Mentha x piperita *L*-menthol				*Syzygium aromaticum* Eugenol				
1a		mg					mg			
Headspace sampling HS-SPME-GC–MS	PHS-1	516.0				CHS-1	7890			
Exhaustive EO isolation GC–MS	PEO-1	398.2				CEO-1	6588			
Residual distillation waters (Rs) SBSE-GC–MS	PR1	23.7				CR1	600			
	PR2	9.6				CR2	316			
	PR3	3.9				CR3	130			
	Total free	37.2					1046			
1b		mg					mg			
EO isolated after EH GC-MS	EH-PEO	490.1				EH-CEO	7120			
EO isolated after EH of Rs GC-MS	PEO-R1-EH	n.q.				CEO-R1-EH	1009			
	PEO-R2-EH	n.q.				CEO-R2-EH	52.5			
		ppm	Free + bound mg	Glucoside mg			ppm	Free + bound mg	Glucoside mg	
Residual distillation waters (Rs) SBSE-GC-MS	PR1-EH	36.8	73.6	50.0		CR1-EH	226	904	304	
	PR2-EH	17.0	33.9	24.3		CR2-EH	115.6	462	146	
	PR3-EH	5.8	11.6	7.7		CR3-EH	46.8	188	58	
	Total		119.1	82		Total		1554	508	
		Glucoside mg					Glucoside mg			
Glucoside in Rs Direct LC–MS	PR1	n.q.				CR1	330.8			
	PR2	n.q.				CR2	158			
	PR3	n.q.				CR3	64			
	Total	n.q.				Total	552.8			
1c		Free mg			Bound mg		Free mg			Bound mg
	Headspace	EO	Rs	R-EH	R	Headspace	EO	Rs	Rs-EH	R
	516	398.2	37.2	119.1	82	7890	6588	1046	1554	508

quantitation, in HS, EO and R, are reported in Table 1SM. Repeatability data for the various approaches and methods adopted in this study are collected in Table 2SM and always acceptable with RSD% ranging between 0.9 to 7.6 for *L*-menthol and 1.2–6.9 for eugenol.

2.1. Peppermint aerial parts

2.1.1. Determination of free L-menthol

2.1.1.1. Quantitation of L-menthol in the headspace of peppermint aerial parts. The total area of *L*-menthol was estimated through three consecutive MHS-SPME extractions of a suitable amount of peppermint aerial parts. The resulting decay equation of *L*-menthol in peppermint, linear regression coefficient average quotient *Q* value are reported in Table 1SM. A calibration curve was built up by analysing a set of *L*-menthol standard solutions under the same conditions (i.e. three consecutive extractions) as reported in paragraph 4.3.5. The resulting amount of *L*-menthol in peppermint aerial parts, determined by MHS-SPME-GC–MS, was 516.0 mg, with an RSD of 3.4% (PHS-1) (Table 1a).

2.1.1.2. L-Menthol in peppermint essential oil. Table 2 reports the list and relative percentage abundances normalised *versus* α-thujone of hydroxylated components of interest for this study. The GC-FID profile of the EO obtained by conventional hydrodistillation (HD) from peppermint aerial parts (PEO-1) and list of all identified components and the chemical composition of PEO expressed as relative percentage abundances are included in supplementary material (Fig. 2SM and Table 3SM). Twenty-nine components were identified, the most abundant of them being *L*-menthol (51.2%), *L*-menthone (17.1%) and 1,8-cineole (5.0%). The EO obtained from 10 g of plant material, submitted to micro-hydrodistillation in the dedicated apparatus (PEO-1.1) (Bicchi et al., 1983) gave yield and composition comparable to those of PEO-1 (Table 3SM). These results meant that micro-hydrodistillation (micro-HD) could be applied to all experiments involving enzymatic hydrolysis (EH), and, as a consequence, experiments could be run with comparable volumes resulting from consistent amounts of plant material.

Processed plant material re-submitted to two successive hydrodistillations with "new" water did not produce a measurable EO amount in either case, meaning that it had been exhaustively isolated in the first step. Conversely, the resulting PR-2 and PR-3 were processed together with PR-1 as reported below.

A non-quantifiable amount of peppermint EO, resulting from the distillation of the waters residual from micro-hydrodistillation after enzymatic hydrolysis (PEO-R1-EH), was recovered with cyclohexane and analysed by GC-FID/MS, giving a significant profile. Twenty-nine compounds were identified in PEO-R1-EH, some of them not found in PEO-1 or EH-PEO – chiefly 3-hexenol (0.09%) – some others absent in PEO-R1-EH, although present in both PEO-1 and EH-PEO (e.g. *trans*-sabinene hydrate). Conversely, adequate amounts of PEO-R2-EH and PEO-R3-EH to carry out

Table 2
Normalised % composition *versus* α-thujone of peppermint (PEO) and clove (CEO) essential oils after GC-FID analysis. PEO-1/CEO-1: peppermint/clove EO obtained with conventional hydrodistillation from 100 g of leaves; PEO-1.1/CEO-1.1: peppermint/clove EO obtained by micro-hydrodistillation from 10 g of leaves; EH-PEO/EH-CEO: peppermint/clove EO obtained from plant material after enzymatic hydrolysis of water-soluble components (micro-hydrodistillation – 10 g); PEO-R1-EH/CEO-R1-EH: peppermint/clove EO obtained from residual distillation waters (Rs) after enzymatic hydrolysis (micro-HD – 200 ml of R1).

		Mentha × *piperita*							
		PEO-1		PEO-1.1		EH-PEO		PEO-R1-EH	
Components	LRI	Av. %	RSD %	Av. %	RSD %	Av. %	RSD %	Av. %	RSD %
3-Hexenol		/	/	/	/	/	/	0.1	2.4
3-Octanol	1419	0.2	2.7	0.2	3.6	0.8	1.6	2.0	0.1
1-Octenol	1423	0.1	4.7	/	/	0.8	2.7	1.8	1.5
i-Pulegol	1549	0.1	4.0	0.1	8.0	0.1	13.7	0.1	1.8
neo-Menthol	1557	4.3	0.3	4.1	1.3	4.2	0.7	4.0	1.1
neo-*i*-Menthol	1646	1.2	0.9	1.1	3.5	1.2	0.8	1.1	1.0
L-menthol	1654	51.2	0.05	52.1	0.1	52.5	0.04	50.7	0.1
Viridiflorol	2081	1.3	0.7	1.3	2.9	1.2	1.2	2.0	0.1
		Syzygium aromaticum							
		CEO-1		CEO-1.1		EH-CEO		CEO-R1-EH	
Components	LRI	Av. %	RSD %	Av. %	RSD %	Av. %	RSD %	Av. %	RSD %
Eugenol	2169	80.3	0.006	81.2	0.1	88.8	1.3	91.7	0.6

qualitative analysis could not be recovered. Table 1a–c is a synopsis of the free and glucosidically-bound *L*-menthol quantitative results, expressed as mg of *L*-menthol in 100 g of plant material. PEO-1 and PEO-1.1 were obtained in a yield of 0.75%, i.e. 750 mg of EO, corresponding to 398.2 mg of *L*-menthol; this was confirmed by GC-FID/MS (Table 1a).

2.1.1.3. Quantitation of free L-menthol in peppermint residual distillation waters (PR). *L*-menthol was first analysed in the residual distillation waters by SBSE-GC-FID/MS. Free *L*-menthol concentration accounted for 11.8 ppm in PR-1, corresponding to 23.7 mg, while its concentration in PR-2 was 4.8 ppm, corresponding to 9.6 mg, and in PR-3 it was 2.0 ppm, corresponding to 3.9 mg (Table 1a).

The above results were confirmed by in-solution SPME-GC-FID/MS applied to all PR samples (data not reported). Moreover, *L*-menthol determined by HS-SPME-GC–MS, and that resulting from the sum of its contents in the essential oil and as free form in the residual distillation waters, were in a good agreement (Table 1c).

2.1.2. Determination of glucosidically-bound L-menthol

2.1.2.1. Peppermint aerial part essential oil after enzymatic hydrolysis. The same twenty-nine compounds as for PEO-1 and PEO-1.1 were identified in the essential oil obtained by micro-hydrodistillation, after hydrolysis of the water-soluble components of peppermint aerial parts with β-glucosidase, although, as expected, the abundance normalised *versus* α-thujone of some alcohols increased, due to enzymatic hydrolysis, compared to PEO-1.1; these included 3-octanol, 1-octenol, and *L*-menthol (Table 2).

The enzymatic hydrolysis effect on plant material suspended in water, and on the residual distillation water, was evaluated through both the abundance of components in their hydrodistillation products (Table 2), and the normalised area ratios of those components that can exist in glucosidic form (alcohols) *vs.* menthone, i.e. a components whose abundance should not vary when submitted to enzymatic hydrolysis (Table 3). Comparison of PEO-1 and EH-PEO results showed that only alcohols/menthone ratios increased because of enzymatic hydrolysis, although to different extents because of their different amounts in the original matrix, while 1,8-cineole/menthone and menthyl acetate/menthone ratios were almost constant, as expected, these two compounds not being involved in enzymatic hydrolysis. The EO yield after preliminary enzymatic hydrolysis (EH-PEO) was 0.93%, i.e. 930 mg of EO, in its turn corresponding to 490.1 mg of *L*-menthol (Table 1a). The additional amount of *L*-menthol deriving from enzymatic hydrolysis in combination with hydrodistillation was therefore 91.9 mg.

Table 3
Main components/menthone I.S.-normalised area ratios in the peppermint EOs obtained under different conditions. PEO-1.2: peppermint EO from micro-hydrodistillation; EH-PEO: peppermint EO from micro-hydrodistillation after hydrolysis of plant material suspended in water with β-glucosidase.

	Mentha × *piperita* Essential oils	
Analyte ratios*	PEO-1.1 (mean ± SD)	EH-PEO (mean ± SD)
1,8-Cineole/menthone	0.3437 ± 0.0014	0.3548 ± 0.0012
3-Octanol/menthone	0.0097 ± 0.0004	0.0495 ± 0.0007
1-Octenol/menthone	0.0047 ± 0.0002	0.0531 ± 0.0014
Menthyl acetate/menthone	0.2535 ± 0.0009	0.2704 ± 0.0012
Neomenthol/menthone	0.2480 ± 0.0033	0.2636 ± 0.0018
Neoisomenthol/menthone	0.0692 ± 0.0024	0.0766 ± 0.0009
L-menthol/menthone	3.0056 ± 0.0026	3.3014 ± 0.0129
Viridiflorol/menthone	0.0866 ± 0.0026	0.0786 ± 0.0013

2.1.2.2. Quantitation of glucosidically-bound L-menthol in peppermint residual distillation waters (PR). The aqueous extracts resulting from enzymatic hydrolysis (EH) were analysed by SBSE-GC-FID/MS. Table 4 reports the areas of the PR1, PR2 and PR3 main components, normalised *vs.* the α-thujone internal standard, after enzymatic hydrolysis, together with their% increase compared to areas for aglycones. These results clearly show that enzymatic hydrolysis markedly increases the abundance of hydroxylated aglycones in PR1-EH, PR2-EH and PR3-EH, while keeping the amount of carbonyl derivatives constant. Some alcohol components were only present after enzymatic hydrolysis, e.g. 3-octanol, linalool, α-terpineol, benzyl alcohol, *p*-menthadienol in PR1-EH, while 3-octenol was only detected in PR2-EH. Further components not found in the EOs were also identified, e.g. benzyl alcohol, *p*-menthadienol.

Glucosidically-bound *L*-menthol was quantitated by applying the same conditions as for the aglycone. Its concentration accounted for 36.8 ppm in PR1-EH, corresponding to 73.7 mg, while its concentration in PR2-EH was 17.0 ppm that is 33.9 mg, and in PR3-EH it was 5.8 ppm that is 11.6 mg (Table 1b). The above results were confirmed by in-solution-SPME-GC-FID/MS applied to all PR-EH samples.

These analyses showed that the total *L*-menthol deriving from enzymatic hydrolysis of its glucoside, in PR1, PR2 and PR3, accounted for 81.9 mg in 100 g of plant material, which is in agreement with EH-PEO (91.9 mg).

Table 4
Areas normalised *vs.* α-thujone as internal standard and % increase of the peppermint and clove residual distillation water main components before and after enzymatic hydrolysis resulting from SBSE-GC-FID analysis. PR1, PR2 and PR3: peppermint first, second and third residual distillation waters, PR1-EH, PR2-EH, PR3-EH: peppermint first, second and third residual distillation waters after enzymatic hydrolysis. CR1, CR2 and CR3: clove first, second and third residual distillation waters, CR1-EH, CR2-EH, CR3-EH: clove first, second and third residual distillation waters after enzymatic hydrolysis.

	Mentha × piperita														
Compounds	PR1	RSD %	PR1-EH	RSD %	% Increase	PR2	RSD %	PR2-EH	RSD %	% Increase	PR3	RSD %	PR3-EH	RSD %	% Increase
1,8-Cineole	0.1	13	0.1	18	−19	0.04	6.3	0.03	8.7	−13	0.02	6.0	0.01	8.7	−50
Menthone	0.3	5.7	0.2	10	−45	0.04	0.9	0.05	0.4	12	0.01	1.5	0.01	0.4	10
Neo-menthol	0.1	1.9	0.2	10	167	0.03	7.2	0.2	1.7	529	0.01	6.9	0.1	1.7	800
Neo-*i*-menthol	0.02	2.0	0.03	12	80	0.01	15	0.02	3.9	119	/	/	/	/	/
L-menthol	0.7	3.7	2.0	3.9	176	0.3	8.8	1.0	1.1	226	0.12	7.2	0.35	1.1	192
Viridiflorol	0.01	3.5	0.02	0.4	26	/	/	/	/	/	/	/	/	/	/
	Syzygium aromaticum														
Compounds	CR1	RSD %	CR1-EH	RSD %	% increase	CR2	RSD %	CR2-EH	RSD %	% increase	CR3	RSD %	CR3-EH	RSD %	% increase
t-Caryophyllene	0.01	4.0	0.01	1.3	−21	0.01	1.0	0.004	8.9	−58	/	/	/	/	/
Eugenol	1.4	0.02	1.8	0.5	30	0.8	0.1	1.0	0.8	27	0.3	1.2	0.4	1.5	35
Chavicol	0.03	0.4	0.03	7.3	18	0.06	5.4	0.04	0.6	−44	0.01	2.5	0.01	1.9	10

2.1.2.3. LC-MS analysis of L-menthol glucoside in peppermint residual distillation waters (PRs). *L*-Menthol glucoside in PR1, PR2 and PR3 was identified by direct injection of the PRs in an HPLC-MS-MS system with a triple quadrupole analyser, through its fragmentation pattern and by comparison with a 78% pure standard from the authors' standard collection of reference compounds and extracts. The ESI + precursor ion was 341.00 m/z $[M + Na]^+$ while the transitions monitored were *m/z* 341.00 → 179.00 and *m/z* 341.00 → 161.00, these two ions respectively corresponding to the adducts [*L*-menthol (*m/z* 156) + Na]$^+$ and [*L*-menthol – H_2O + Na (*m/z* 161)]$^+$. The amount of *L*-menthol glucoside in PRs was not reliably measurable, making its quantitation unsuitable for consideration for comparison with the other methods applied.

2.2. Cloves

2.2.1. Determination of free Eugenol

2.2.1.1. Quantitation of eugenol in the headspace of cloves. Eugenol was quantitated in cloves by MHS-SPME-GC–MS, with three consecutive extractions of 1 mg of cloves dispersed in Celite at a 1:20 ratio, to achieve linear decay. Decay equation, linear regression coefficient and average quotient Q of eugenol in cloves are collected in Table 1SM. A calibration curve was built up by analysing a set of eugenol standard solutions under the same conditions (i.e. three consecutive extractions) as reported in paragraph 4.3.5. The amount of eugenol in clove, determined by MHS-SPME-GC–MS (CHS-1), was 78.9 mg per g of plant material, corresponding to 7890 mg in 100 g of cloves (Table 1a).

2.2.1.2. Eugenol in clove essential oil. The same procedure adopted for peppermint aerial parts was applied to cloves. Table 2 reports the list and relative percentage abundances normalised *versus* α-thujone of eugenol in clove. The GC-FID profile of the EO obtained by conventional hydrodistillation (HD) from clove (CEO-1) and list of all identified components and the chemical composition of CEO expressed as relative percentage abundances are included in supplementary material (Figure 3SM and Table 4SM). Five components were identified, the most abundant of them being eugenol (80.3%), eugenyl acetate (10.3%) and *trans*-β-caryophyllene (8.1%). Table 1a–c lists the quantitative results for eugenol, expressed as mg of eugenol in 100 g of plant material. As for *L*-menthol in peppermint aerial parts, processed plant material was re-submitted to two successive hydrodistillations with "new" water, without obtaining a measurable EO amount in either case. The resulting CR2 and CR3 were processed together with CR1, as indicated below.

The yield of the essential oil obtained by hydrodistillation for the investigated clove sample (CEO-1) was 8.8 g of EO in 100 g of plant material, corresponding to 6588 mg of eugenol; these results were confirmed by quantitation through GC-MS (Table 1a).

2.2.1.3. Quantitation of free eugenol in clove residual distillation waters (CR). Free eugenol was first quantitated in CR1, CR2 and CR3 by SBSE-GC-FID/MS; the results are summarised in Table 1a. Free eugenol concentration in CR1 was 150 ppm, corresponding to 600 mg, while its concentration in CR2 was 78.9 ppm, i.e. 316 mg, and in CR3 it was 32 ppm, i.e. 130 mg. These analyses showed that the residual amount of free eugenol in CR1, CR2 and CR3 was quite significant (about 13%, i.e. 1046 mg). In this case, too, the results for eugenol were confirmed by in-solution-SPME-GC–MS (data not reported). Moreover, the amount of free eugenol contained in the essential oil and in the residual distillation waters was in a good agreement with that determined by HS-SPME-GC–MS in cloves. (Table 1c).

2.2.2. Determination of glucosidically-bound eugenol

2.2.2.1. Clove essential oil after enzymatic hydrolysis. The clove EO obtained after enzymatic hydrolysis (EH-CEO) gave a 11.6 g of EO in 100 g of cloves, corresponding to 7120 mg of eugenol (Table 2a). The amount of eugenol deriving from the combination enzymatic hydrolysis-hydrodistillation was therefore 532 mg. The same five compounds as for CEO-1 were identified and monitored, but the GC-FID/MS profile changed, especially for eugenol, which accounted for 88.8% of the EH-CEO composition.

2.2.2.2. Quantitation of glucosidically-bound eugenol in clove residual distillation waters (CR). Glucosidically-bound eugenol was quantitated in CR-1, CR-2 and CR-3 by SBSE-GC-FID/MS after enzymatic hydrolysis under the same conditions reported for the aglycone. The eugenol quantitative results are summarised in Table 1b. Eugenol concentration in CR-1 after enzymatic hydrolysis (CR-1-EH) was 226 ppm, corresponding to 904 mg, while its concentration in CR-2-EH was 115.6 ppm, i.e. 462 mg, and in CR3-EH it was 46.8 ppm, i.e. 188 mg.

Table 4 reports the areas of the main components of CR1, CR2 and CR3 and CR1-EH, CR2-EH and CR3-EH i.e. before and after enzymatic hydrolysis, normalised *vs.* α-thujone as internal standard, together with their % increase. These results clearly show the increase of eugenol content in CR1 and CR2 due to enzymatic hydrolysis, whereas the amount of hydrocarbons remained constant. In this case too, the results were confirmed by in-solution-SPME-GC-FID/MS (data not reported).

Again, eugenol determined by HS-SPME-GC–MS and that resulting from the sum of its contents in the essential oil and in the residual waters were in a good agreement.

Unlike peppermint aerial parts, hydrodistillation of clove residual distillation waters after enzymatic hydrolysis (CEO-R1-EH and CEO-R2-EH) produced measurable essential oil yields: (a) CEO-R1-EH yield was 1.1%, calculated on the initial mass of plant material, and contained 91.7% of eugenol, corresponding to 1009 mg, while (b) for CEO-R2-EH, it was 0.055% and contained 95.1% of eugenol, corresponding to 52.5 mg (Table 1b). Conversely, the amount of CEO-R3-EH was negligible, and could not be recovered for analysis.

2.2.2.3. LC–MS analysis of eugenol glucoside in clove residual distillation waters (CRs). Eugenol glucoside was identified by its UV spectra (maximum at 276 nm) and mass spectral data in MRM mode in both ESI+ and ESI−). The MRM transitions were selected on the basis of the fragment ions obtained by analyzing the eugenyl glucoside standard, using 349.00 m/z $[M + Na]^+$ for ESI+ and 371.00 m/z $[M + \text{Formic Acid-H}]^-$ for ESI− as precursor ions. The monitored transitions were: (a) for ESI+: *m/z* 349.00 → 187.00 corresponding to the adduct [eugenol (*m/z* 164) + $Na]^+$, (b) for ESI-: *m/z* 371.00 → 163.00 corresponding to the fragments [eugenol (*m/z* 164) – $1]^-$. The quantitation of eugenyl glucoside in CRs was carried out on the PDA-UV profiles (at 276 nm) using the external calibration method. These analyses confirmed its presence in the three CRs in amounts very similar to those obtained indirectly after enzymatic hydrolysis (Table 1b).

3. Conclusions

The results clearly show that the amount of characterising markers, in both free and glucosidically-bound forms that is not recovered when an EO is isolated by hydrodistillation is quite considerable. The aglycone(s) not recovered by hydrodistillation can indicatively be calculated as a percentage through the following equation:

$$Q_{HS} - Q_{EO}/Q_{HS} \times 100 \quad (3)$$

where Q_{HS} is the quantity naturally occurring in the plant material measured through headspace (HS) and Q_{EO} is the amount recovered from the EO. In the matrices investigated here, these percentages accounted for 22.8% for *L*-menthol in peppermint aerial parts, and 16.5% for eugenol in cloves. Of these two compounds, a significant amount is solubilised as aglycone in the residual distillation waters (R); in the investigated matrices, free *L*-menthol in R accounted for 7.2% of the HS reference amount, and free eugenol for 13.3%; at the same time, the dissolved aglycone represents 9.3% and 15.9% compared to the amounts in their respective EOs. The differences in percentages between the two markers, in the two plant matrices investigated, are fully explained by their significantly different water solubility. The amount of related glucosides are, of course, not included in the HS-determined aglycone, although they may provide an extra yield of the marker if submitted to hydrolysis. In this case, *L*-menthol from its glucoside is 20.6% of that in the EO of peppermint aerial parts, while eugenol deriving from its glucoside accounted for 7.7% of the eugenol in clove EO. Another possibility to increase the yield of the investigated markers is to submit plant material to enzymatic hydrolysis prior to hydrodistillation: when this was done, the increase of *L*-menthol accounted for 23.1%, and that of eugenol for 8.1%, compared to the amount in the EOs without enzymatic hydrolysis. These results indicates that the residual distillation waters are by-products exploitable as a source of useful compounds; this is in line with today's general trends aiming at reducing the dissipation of active components in the environment to a minimum, and at exploiting plant resources exhaustively.

The strategy adopted here also aimed to evaluate the impact of recently-introduced techniques that are rarely applied to investigations in this field, if at all. The combined use, for the first time, of high-concentration-capacity sample preparation techniques (SBSE, and HS-SPME and in-solution SPME) making it possible to run reliable quali-quantitative analysis directly, i.e. without sample manipulation, and direct LC–MS glucoside analysis, provided cross-validation and increased the reliability of the reported results. This strategy has been shown to be reliable, since the quantitative results obtained by different approaches agreed: e.g. (1) the sum of the marker amounts contained in the EO and the total aglycones in R, compared to the amount determined by HS sampling, accounted respectively for 84.4% of *L*-menthol and for 96.8% in the case of eugenol, and (2) with cloves, the amount and percentages of glucosides expressed as eugenol was closely consistent for EH-CEO (532 mg), CR-EH (508 mg) and by direct CR LC–MS (552 mg), varying by up to 8.0%. Similar results were obtained with peppermint aerial parts, where the glucoside amounts were closely comparable, being 92.1 mg for EH-PEO, and 82 for PR-EH, i.e. differing by about 11%.

4. Experimental

4.1. Plant material

Plant material: *Mentha x piperita* L. (Lamiaceae). Aerial parts of peppermint were harvested in 2012 and dried at room temperature in the shade; they were kindly supplied by Dr. Franco Chialva (ChialvaMenta, Pancalieri, Turin Italy).

Dried cloves (*Syzygium aromaticum* (L.) Merr. & L.M.Perry, Myrtaceae) were purchased from Cannamela (Zola Predosa (BO), Italy). Cloves were finely powdered before analysis.

4.2. Reagents and chemicals

All chemicals were analytical grade. Octyl-β-D-glucopyranoside, β-glucosidase and Bis-(trimethylslyl)-trifluoroacetamide (derivatization grade) were from Sigma Aldrich (Milan, Italy). α-Thujone and eugenol (99%) were from Sigma Aldrich (Milan, Italy). Silica gel 60 (70–230 mesh) was from Macherey–Nagel. Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, visualised by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. Organic phases were dried with Na_2SO_4 before evaporation. Solvents (acetic acid, dichloromethane, ether, ethyl acetate, methanol, petrolether, tetrahydrofuran) and other reagents were from Sigma Aldrich. All solvents were dried following standard procedures.

4.3. Sample preparation and analysis

Each step of the procedure described in the following paragraphs was repeated three times, and the mean areas of the GC-FID and GC–MS analysis were considered for further processing. All steps are summarised in Fig. 1.

4.3.1. Isolation of essential oils (EOs)

EOs were isolated from 100 g of homogeneous peppermint aerial parts, and from 50 g of powdered cloves, by hydrodistillation using 2000 mL of distilled water. Conventional hydrodistillation (HD) was performed in a Clevenger-type apparatus for four hours as indicated in the European Pharmacopoeia (2008). The resulting EO was separated from the aqueous layer, dried over anhydrous sodium sulphate, and stored in a sealed vial at low temperature

(−18 °C) until analysis. A similar procedure was applied with a micro-hydrodistillation apparatus (micro-HD) developed in the authors' laboratory, in which 10 times less plant material (i.e. 10 g peppermint aerial part and 5 g cloves) were submitted to hydrodistillation (Bicchi et al., 1983).

4.3.2. Glucosidically-bound volatile extraction

The investigated glucoside fractions were solubilised in the aqueous phase during the hydrodistillation process (HD) to isolate the EOs. The resulting aqueous phase was separated by decantation (first residual distillation-water, R1); the residual plant material was re-suspended in another 2 l of water and re-hydrodistilled, obtaining the second essential oil (EO-2) and the second residual distillation water (R2). This operation was repeated a third time under the same conditions, to give the third essential oil (EO-3) and the third residual distillation water (R3).

4.3.3. Isolation of essential oils after enzymatic hydrolysis

The plant materials were also submitted to enzymatic hydrolysis (EH) prior to hydrodistillation, roughly following the method described by Zhiping et al. (2006) to isolate the volatile fraction from roses. 10 g of plant material from *Mentha x piperita* and 5 g from *Syzygium aromaticum* were suspended in a buffer solution (pH 5.5) consisting of 250 ml distilled water, 3.608 g anhydrous sodium acetate, and 0.35 ml acetic acid, to which 0.1 g β-glucosidase from almonds were added. The activity of β-glucosidase was ⩾6 U/mg. The resulting sample was kept at 37 °C overnight under stirring, and subsequently submitted to micro-hydrodistillation in the above microapparatus (Bicchi et al., 1983) as described in paragraph 4.3.1, to give the corresponding essential oil (EH-EO).

4.3.4. Glucoside hydrolysis in the residual distillation waters (R)

Acidic and enzymatic hydrolysis procedures were experimented directly on the residual water samples resulting from hydrodistillation (after pH adjustment). Preliminary hydrolysis assays were carried out using a standard of octyl-β-glucoside as model compound, to tune both the acidic and the enzymatic hydrolysis conditions of glucosidically-bound volatiles.

4.3.4.1. Acidic hydrolysis. Diluted hydrochloric acid was added to 4 ml of R1, to pH 3.0 and to pH 1.0, in sealed vials, and then heated for 60 min at 100 °C (Maicas and Mateo 2005).

4.3.4.2. Enzymatic hydrolysis. β-glucosidase (16 mg) was added to 4 ml of R1, at pH 5.5, in a sealed vial and submitted to incubation under stirring at 37 °C overnight. Enzymatic hydrolysis conditions were in agreement with those reported in the existing literature (Morales et al., 2000; Boulanger and Crouzet, 2001; Mastelic and Jerkovic, 2003; Radonic and Mastelic, 2008). The incubation times giving the highest hydrolysis yield (99 to 100%) ranged from 12 to 24 h while free *L*-menthol or eugenol in PR1 and CR1 under the same conditions did not increase after 18 h.

The hydrolysis process was monitored, taking n-octyl-β-glucoside as internal standard. R1 were first controlled to check whether free 1-octanol and n-octyl-β-glucoside were absent. These operations were repeated on R2 and R3. The hydrolysed volatile aglycones were recovered from the aqueous phase, by hydrodistillation and solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) operating both in headspace and in-solution modes (for analysis conditions par. 4.3.5 and 4.3.6).

4.3.5. Headspace (HS) analysis of the volatile fraction

The HS composition of peppermint dried leaves, (*Mentha x piperita*), and cloves (*Syzygium aromaticum*) was determined by headspace solid phase microextraction (HS-SPME) sampling with a DVB/Carboxen/PDMS (2 cm) fibre.

SPME device and CAR/PDMS/DVB fused silica fibres were supplied by Supelco (Bellafonte, PA, USA). Before use, all fibres were conditioned as recommended by the manufacturer, and tested to evaluate the consistency of their performance *versus* reference peppermint and clove samples. In addition, fibre extraction reliability was evaluated by means of full evaporation HS-SPME sampling of the vapor phase from a standard solution of α-thujone (5 μl of a 2000 ppm solution in di-butyl phtalate – DBP) and of 1 μl of a 5000 ppm peppermint EO solution in DBP (Bicchi et al., 2007).

4.3.5.1. Headspace sampling conditions. 1 mg of plant material and 10 μl of a 10 ppm solution of α-thujone in 1:1 water:ethanol (v/v), used as internal standard, were submitted to HS-SPME sampling with a CAR/PDMS/DVB fused silica fibre (2 cm–50/30 μm) for 30 min at 35 °C.

4.3.5.2. Multiple headspace solid phase microextraction (MHS-SPME). *L*-menthol and eugenol were quantitated with the multiple headspace extraction approach (MHS-SPME). The total area of eugenol and menthol was estimated with three consecutive samplings of a suitable amount, 1 mg of peppermint aerial part sample, and 1 mg of a mixture of powdered cloves and Celite (1:20, w/w), in order to obtain correct decay trend. A calibration curve was built up by analysing two sets of solutions of *L*-menthol and eugenol in acetone under the same conditions (i.e. three consecutive extractions). Individual stock solutions of *L*-menthol and eugenol were prepared in a 20 ml vial by adding 500 mg of pure standard to an appropriate volume of HPLC grade acetone (10 ml) to obtain analyte concentrations of about 50000 and 15000 ppm, respectively. The calibration solutions of *L*-menthol were 7500, 5000, 3000 and 1000 ppm, while those of eugenol were obtained by diluting the stock solution to 25000, 10000, 5000 and 2500 ppm. The calibration curve was built up by introducing 1 μl of each solution in a 20 ml vial and submitting each vial to three consecutive extractions (in triplicate for each concentration). The resulting standard solutions were stored at 0 °C and renewed weekly (Bicchi et al., 2011).

4.3.6. SBSE and in-solution-SPME

SBSE for in-solution-sampling was run with commercial PDMS-EG twisters™ (d_f 500 μm–2 cm) supplied by Gerstel (Mülheim a/d Ruhr, Germany). Sampling was carried out in a thermostatic bath.

For SBSE sampling, twisters were introduced into the sample solution in a 10 ml vial under the following conditions: sample amount: 4 mL of R containing 10 μL of a 10 ppm solution of α-thujone in water:ethanol, 1:1 (v/v), used as internal standard; temperature: 35 °C; sampling time: 15 min with agitation at 250 rpm (Bicchi et al., 2002). Quantitation of *L*-menthol and eugenol was done by GC-FID and GC–SIM–MS from the corresponding calibration curves, built within a suitable range of concentrations, i.e. 10 to 250 ppm for menthol, and 25 to 500 ppm for eugenol resulting from the dilution of a *L*-menthol and eugenol solutions in acetone (HPLC grade) with a suitable volume of HPLC water. The following target ions and qualifiers were chosen: *L*-menthol target ion: 71 m/z, qualifiers 123 and 138 m/z, eugenol: 164 m/z, qualifiers 149 and 77 m/z.

In-solution SPME was carried out under the same conditions as for SBSE, adopting a CAR/PDMS/DVB fibre.

4.3.7. Analysis conditions

Each sample was analysed in triplicate, using α-thujone as internal standard, and the FID mean area values for area % normalisation and SIM-MS peak area for quantitative analysis.

GC–MS analyses were carried out with an Agilent 6890 unit provided with FID combined with a mass selective detector model 5973 N. GC operative conditions were:

(a) EOs were analysed by injecting 1 μl of a solution diluted 1:200 in cyclohexane through a MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr, Germany) on-line combined with the above GC–MS system in split mode, split ratio 1:20.
(b) In HS-SPME and in-solution-SPME, the sampled volatiles were recovered by direct desorption (10 min at 230 °C) in the GC injector port through a MPS-2 multipurpose sampler on-line combined with the above GC–MS system in split mode, split ratio 1:20.
(c) In HSSE and SBSE, volatiles were on-line transferred to GC–MS by a MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr, Germany) equipped with a Thermo Desorption Unit (TDU) and a CIS-4 PTV injector (Gerstel, Mülheim a/d Ruhr, Germany).

TDU operative conditions: from 30 °C to 270 °C (5 min) at 60 °C/min; flow mode: splitless; transfer line: 270 °C, CIS-4 PTV injector temp: −50 °C; coolant: liquid CO_2; injection temperature program: from −50 °C to 270 °C (10 min) at 12 °C/s; inlet operated in split mode: split ratio 1:20.

EOs, HS, and in-solution sampled samples were analysed by GC with a polyethylene glycol fused silica column (50 m × 200 μm × 0.200 μm i.d.) (MEGA-WAX MEGA Legnano, Milan, Italy). Injector and FID detector temp. were maintained at 250 °C and 280 °C, respectively. The column oven was programmed from 35 °C (1 min) to 250 °C (5 min) at 3 °C/min. Carrier gas: (He) flow-rate: 1 mL/min; injection mode: split, ratio: 1:20. MS conditions: ionisation mode EI at 70 eV; transfer line temp.: 250 °C; ion source temp.: 250 °C; mass range: 35–350 mass units.

Individual peaks were identified by comparing their linear retention indices calculated *versus* a C9–C25 hydrocarbon mixture to those of authentic samples, as well as by comparing their mass spectra to a set of commercial and in-house libraries. Percentage composition was determined from GC-FID data through the peak area normalisation approach, adopting a response factor for each class or sub-class of compounds (hydrocarbons, aldehydes, alcohols, esters, etc.) in the investigated sample, calculated *versus* the internal standard, taking one component representative of each class (Costa et al., 2008).

4.4. Repeatability of the method

Repeatability was determined in different ways depending on the sample analysed: (a) MHS-SPME of plant material: three samples of each matrix were submitted to MHE for a total of 9 analyses; (b) each EO was analysed three times by split injection-GC-FID after a 1:200 dilution in cyclohexane; (c) each R was sampled three times by SBSE and analysed by GC–MS. Repeatability was evaluated on *L*-menthol for peppermint aerial parts and on eugenol for clove; it is expressed as Relative Standard Deviation% (RSD%) on normalised peak areas of *L*-menthol and eugenol *versus* α-thujone as internal standard.

4.5. Eugenol glucoside synthesis

The synthetic pathway is reported in Fig. 1SM (supplementary material).

4.5.1. Synthesis of α-acetobromoglucose

α-Acetobromoglucose (2,3,4,6-tetra-O-acetyl-D-glucopyranosyl bromide, MW = 410.02) was prepared from glucose penta-acetate (MW = 390.12; 7.69 mmol) dissolved in 10 mL of dichloromethane and hydrogen bromine 33% in acetic acid (MW = 80.12; 38.46 mmol). The reaction was run for 6 h under stirring at room temperature and monitored by TLC. Brine and sodium bicarbonate were then added; the organic phase was extracted with dichloromethane, dried with sodium sulphate, filtered and recrystallised from ether/petroleum ether 1:1 (yield: 86%). The product was used without any further purification.

4.5.2. Synthesis of eugenyl peracetylglucoside

Eugenyl-2,3,4,6-tetra-O-acetyl-D-glucopyranoside (Mw = 494.18) was prepared by reacting α-acetobromoglucose (0.487 mmol) and eugenol (0.975 mmol) in acetic acid and sodium hydroxide 0.725 N to obtain a pH value around 9. After a few minutes the pH dropped to 7; it was thereafter maintained at around 9 by further additions of sodium hydroxide. The reaction mixture was stirred for 24 h at room temperature and the reaction monitored by TLC. Brine was then added; the organic phase was extracted with tetrahydrofuran, dried with sodium sulphate, filtered, and the resulting eugenyl peracetylglucoside purified by column chromatography with petroleum ether/ethyl acetate 8:2 (yield: 52%). White powder, 1H NMR (300 MHz, $CDCl_3$): δ 7.02 (1H, d, J = 7.95 Hz, 1'-H), 6.70 (1H, s, 3'-H), 6.68 (1H, d, J = 8.25 Hz, 2'-H), 5.90 (1H, m, 5'-H), 5.70 (1H, d, J = 8.28 Hz, 1-H), 5.30–5.10 (3H, m, 2-, 3- and 4-H), 4.89 (2H, m, 6'-H), 4.25–4,06 (2H, dd, 6-H-6a,b), 3.81 (3H, s, -OMe), 3.78 (1H, m, 5-H), 3.32 (2H, d, J = 6.12 Hz, 4'-H), 2.10–2.02 (12H, s, 2-, 3-, 4-, 6-OAc).

4.5.3. Synthesis of eugenyl glucoside

Eugenyl peracetylglucoside (0.243 mmol) was dissolved in methanol (4 mL) and deacetylated with sodium methoxide 0.5% in methanol. The reaction was stirred at room temperature for 1 h and monitored by TLC. Brine was then added; the organic phase was extracted with tetrahydrofuran, dried with sodium sulphate, filtered, and the resulting eugenyl glucoside (MW = 326.14) was purified by column chromatography with petroleum ether/ethyl acetate 8:2 and ethyl acetate/methanol 8:2 (yield: 33%). White powder, ^{1}H NMR (300 MHz, DMSO-d6): δ 7.07 (1H, d, J = 7.95 Hz, 1'-H), 6.80 (1H, s, 3'-H), 6.70 (1H, d, J = 8.25 Hz, 2'-H), 5.92 (1H, m, 5'-H), 5.06 (1H, s, -OH), 5.02 (1H, d, 6'-H), 4.88 (1H, m, 1-H), 4.85 (1H, s, -OH), 4.55 (1H, s, -OH), 3.81 (3H, s, OMe), 3.68–3,25 (2H, dd, 6AB-H), 3.44 (2H, d, J = 6.12 Hz, 4'-H), 3.68–3,25 (4H, m, 2–3-, 4- and 5-H).

4.6. HPLC-MS analysis

4.6.1. Analysis conditions

Analyses were carried out on a Shimadzu Nexera X2 system equipped with a photodiode detector SPD-M20A in series to a triple quadrupole Shimadzu LCMS-8040 system provided with electrospray ionisation (ESI) source (Shimadzu, Dusseldorf Germany). An Ascentis® Express C18 column (150 × 2.1 mm i.d., 2.7 μm particle size), (Supelco, Bellefonte, PA) was used. Analysis conditions: mobile phase: eluent A: 0.1% formic acid in water; eluent B: 0.1% formic acid in acetonitrile; mobile phase gradient was as follows: 5–25% B in 10 min, 25–40% B in 5 min, 40–100% B in 5 min, and 100% B for 1 min. Injection volume: 5 μl; flow-rate 0.4 ml/min; column temperature: 30 °C. UV wavelength range: 210–450 nm. MS operative conditions: heat block temperature: 400 °C; nebulizing gas (nitrogen) flow-rate: 3 l/min; drying gas (nitrogen) flow-rate: 15 l/min; desolvation line (DL) temperature: 250 °C. Collision gas: argon (230 kPa).

L-menthol glucoside was identified by comparison to a standard available from the library of standards in the authors' laboratory. *L*-menthol glucoside in PRs was identified by its mass spectral data in positive ionisation mode (ESI+) in Multiple Reaction Monitoring

(MRM) mode. The MRM transitions were selected on the fragments obtained by analysing the *L*-menthol glucoside standard in full-scan mode (range: 150–500 m/z; scan speed: 1000 u/s) and then in product ion scan mode in the range 100–400 m/z (scan speed: 1500 u/s).

Eugenyl glucoside was identified and quantitated *versus* a pure standard synthesised as reported in paragraph 4.5. Its identification was based on its UV spectrum (maximum at 276 nm) and mass spectral data in both positive and negative ionisation mode (ESI+ and ESI) in Multiple Reaction Monitoring (MRM) mode. The MRM transitions were selected on the fragments obtained by analysing the eugenyl glucoside standard in full-scan in both ESI+ and ESI− (range: 150–500 m/z; scan speed: 1000 u/s) and then in product ion scan mode in both ESI+ and ESI− in the range 100–400 m/z, scan speed: 1500 u/s (dwell time: 20 ms, collision energy: 35 V, event time: 0.096 s). Quantitation was done on the PDA-UV profiles (at 276 nm) using the external calibration method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.06.017.

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