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Determination of Fragrance Allergens in Essential Oils and Evaluation of their *in vitro* Permeation from Essential Oil Formulations through Cultured Skin

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Abstract

We developed a high-performance liquid chromatography method using fluorometric detection to simultaneously determine the allergens in fragrances, primarily structurally related allylbenzenes in essential oils. The effect of essential oil carriers—cloves, basil, and cinnamon—on the release and percutaneous absorption of the oils was studied *in vitro* using a cultured epidermal autograft membrane model.

Keywords: Allergens of fragrance; Essential oil formulations; Permeation through cultured skin; Fluorometric detection

Introduction

Research on essential oils is popular; however, almost all studies focus on their extraction, chemical composition, and wide application in the food and cosmetics industries and traditional medicine. Fragrances are widely encountered in our daily environment and are known to be a common cause of allergic contact dermatitis. Quantitative analysis of fragrance substances that are well-known allergens in cosmetic and essential oil products, such as cinnamic alcohol, isoeugenol, and eugenol in ylang-ylang oil and jasminum, have been extensively studied over the past 10 years (Schlede et al., 2008; Schreiner et al., 2008; Villa et al., 2007; David et al., 2006; Tomar et al., 2005; An et al., 2005; Leijs et al., 2005; Cadby et al., 2003). The major analytical methods for analyzing fragrances are gas chromatography, gas chromatography-mass spectrometry (Schulz et al., 2008a; Schulz et al., 2008b; Polzin et al., 2007; Elzaawely et al., 2007; Yu et al., 2007; Bianchi et al., 2007; Kim et al., 2006; Sánchez-Palomo et al., 2005; Peña-Alvarez et al., 2006; Lukić et al., 2006 ; Mitja et al., 2006; Besharati-Seidani et al., 2005; Luan et al., 2005; Hérent et al., 2007), minor liquid chromatography (Villa et al., 2007; Li et al., 2007; Li et al., 2008; Rauber et al., 2005), micellar electrokinetic capillary chromatography (Huhn et al., 2008; Hanson et al., 2005), and vibrational spectroscopy (Schulz et al., 2003). The *in vitro* evaluation of fragrance materials such as camphor, carvone, 1, 8-cineole, linalool, menthol, thujone, menthone, t-anethole, through the human epidermis has already been studied (Gabbanini et al., 2009; Zhang et al., 2006).

Few reports focus on the influence of aromatic essential oils on skin permeation, because the fragrances of these oils are complicated and difficult to characterize (Weyers and Brodbeck, 1989; Weibel and Hansen, 1989; Gabbanini et al., 2009). There are few reports in the literature on using liquid chromatography with fluorometric detection to determine fragrance levels in essential oils and evaluate *in vitro* percutaneous absorption of essential oil formulations through cultured skin. We used the sensitivity and specificity of fluorometric detection to determine fragrance levels, and a cultured epidermal autograft membrane model for an *in vitro* study of the percutaneous absorption of the essential oils; it is more specific because it eliminates interference from natural essential oils.

Experimental

Apparatus and materials

High performance liquid chromatography (HPLC) was done using both a Hitachi model L-7100 pump and model 7125 injector equipped with a 20-μL sample loop and with a fluorometric detector (RF-10AXL; Shimadzu). Chromatograms were acquired and peak areas calculated (D-7000 HPLC System Manager Software; Hitachi). Fragrances with allergens were purchased: cinnamyl alcohol and eugenol methyl ether (Chem Service, Inc., West Chester, PA, USA); eugenol, isoeugenol, and anethole 99% (Acros Organics, Geel, Belgium); α-asarone, myristicin, and safrole (Fluka Chemie AG, Buchs, Switzerland and RDH Laborchemikalien GmbH & Co KG, Seelze, Germany); and 4-Allylanisole (Aldrich Chemical Company, Inc., Milwaukee, WI, USA). The structurally related allergens are shown in Scheme 1. Phosphate buffered saline was obtained from Hyclone (Thermo Fisher Scientific Inc., Waltham, MA, USA). All other chemicals were of analytical reagent grade. Samples of 11 essential oils obtained local department store or retail grocern and drug food cosmetic outlet. They were labeled as 100 % natural products.

Determining fragrances using HPLC

The stationary phase was a Phenomenex C_{18} column (5 μm, i.d.: 4.6×250 mm), and the eluent was a mixture of methanol-water (70:30 (v/v), pH 3.52) and acetonitrile-methanol-water [10:50:40 (*v/v/v*), 20:40:40 (*v/v/v*), 30:30:40 (*v/v/v*), and 40:40:20, (*v/v/v*)], containing 1 mM of phosphoric acid adjusted at pH 3.45, 4.13, 5.13, 6.03, 7.03, and 7.78. The eluent flow rate was 1.0 mL/min, and the fluorometric detector was operated at an Ex (excitation wavelength) of 230 nm and an Em (emission wavelength) of 317 nm. After various analyses of the retention behavior of the fragrances, baseline separation was achieved. Acetonitrile-methanol-water (30:30:40 (v/v/v), pH 6.02) was the best eluent for a good resolution and for the fewest peak interferences in the matrix. Sample and standard solutions (20 μL) were injected using an injection valve.

In vitro skin permeation

The permeation of fragrances *in vitro* was investigated using

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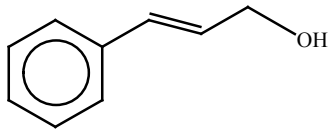
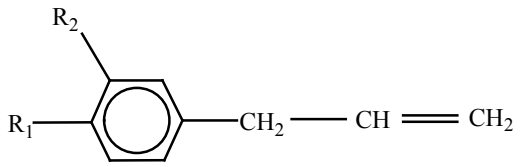
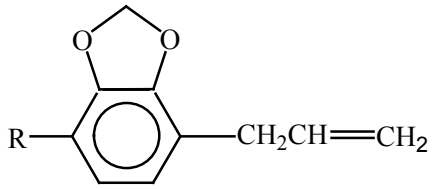


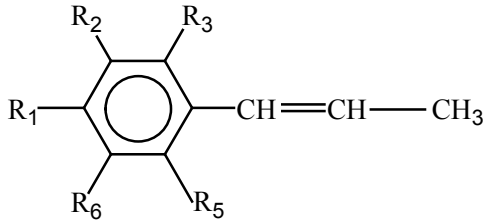
Cinnamyl alcohol



Eugenol $R_1=OH$, $R_2=OCH_3$
Eugenol methyl ether $R_1=OCH_3$, $R_2=OCH_3$
Estragole $R_1=OCH_3$, $R_2=H$



Safrole $R=H$
Myristicin $R=OCH_3$



Anethole $R_2=R_3=R_5=R_6=H$, $R_1=OCH_3$
α-Asarone $R_3=R_5=H$, $R_1=R_2=R_6=OCH_3$
Isoeugenol $R_3=R_5=R_6=H$, $R_2=OCH_3$, $R_1=OH$

Scheme 1: The fragrance structures of cinnamyl alcohol and allylbenzenes derivatives.

a horizontal diffusion cell or a side-bi-side cell constructed in our laboratory (Scheme 2). The diffusion cells consisted of two water-jacketed cylindrical half-cells, each with a permeation area of about 9.6 cm^2. A freshly cultured epidermal autograft membrane cell line (CAL-2309) was obtained from the Animal Technology Institute of Taiwan (http://www.atit.org.tw/english/index_e.htm). Microphotographs of the membranes used for the study were examined for integrity, and then immediately mounted in the diffusion cells. One of the receptor compartments (11 mL) was filled with phosphate buffered saline (PBS) as the cell culture medium; the other contained 0.08% w/v of essential oil micro cream. The less surfactant, the less irritation with topical use can be expected. We investigated four different preparations—A-D—without Tween 80 surfactant (A) but with various amount of Tween 80 surfactants (B-D). To test the effect of essential oil, all preparations (B-D) were mixed with increasing amounts (0.0184%, 0.0245%, and 0.08%) of clove essential oil for B, C, and D preparations, respectively. Emulsifier (surfactant) solubilizes essential oil in water and produces a clear o/w emulsion. A contained 0.0184% clove essential oil without emulsifier (Tween 80), B contained 0.0184% clove essential oil and 0.1% Tween 80, C contained 0.0245% clove essential oil and 0.2% Tween 80, and D contained 0.08% clove essential oil and 0.5% Tween 80 in the receptor The formulation consisted of 9.5 g of water, 0.33 g of Tween 80 (a non-ionic surfactant and emulsifier), 0.17 g of stearic acid, and 0.008 g of essential oil. The diffusion cells were kept at a constant temperature by circulating water at 37°C. The receptor solution was stirred during the experiment to ensure thorough mixing.

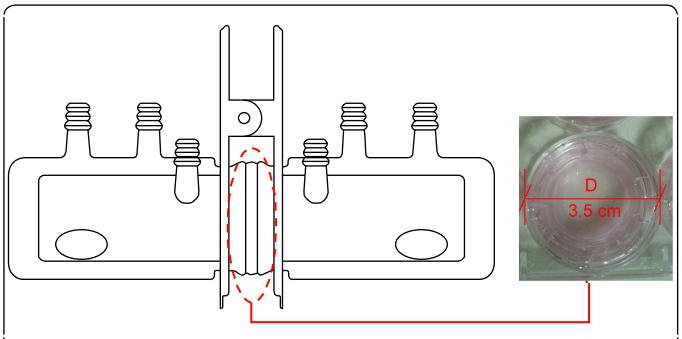
At defined time intervals (0, 10, 20, 30, 60, 90, 120, 180, 240, 360, 420, 480, 600, and 720 min), 1 mL of receptor samples was removed for analysis and replaced with fresh receptor medium. The sample was extracted using three 3-mL portions of n-hexane. The organic phase was collected and evaporated under nitrogen at a temperature < 37°C. Samples were reconstituted with methanol (2 mL), vortex-mixed, and then filtered through 0.45-μm PVDF membrane filters before LC analysis.

Results and Discussion

Optimization of chromatographic conditions

The objective of this study was to develop an HPLC assay for simultaneously determining fragrances in essential oils and in cultured epidermal autograft membrane. Five different eluents with various compositions were prepared and listed in Table 1. With methanol-water (70:30, *v/v*) as the eluent, the retention times of safrole, 4-allylanisole, and trans-anethole were 19.73, 22.45, and 27.37 min, respectively. Table 1 shows the effect of acetonitrile concentration in the mobile phase on the capacity factors and sensitivity. However, acetonitrile-methanol-water (10:50:40 and 20:40:40) failed to resolve the solute eugenol and isoeugenol. The 20:40:40 eluent was too polar for the rapid elution of fragrances. Various pH (3.45-7.78) of acetonitrile-methanol-water (30:30:40, *v/v/v*) were experimented with on fragrances. After various studied of retention behaviour of the fragrances, baseline separation was achieved. Acetonitrile-methanol-water (30:30:40, *v/v/v*, pH 6.02) was found to be the best mobile phase for a good resolution and for the least peak interferences in the matrix. Therefore, it used as the mobile phase and the Phenomenex C_{18} analytical column (particle size 5 μm, 4.6 × 250 mm i.d.) was the stationary phase. A typical HPLC trace under optimum conditions is shown in Figure 1.

The standard curves for all nine compounds were determined simultaneously at Ex 230 nm and Em 317 nm. Using the procedure described, the limits of detection for fragrances were 0.009-0.144 ng mL^{-1}. All calibration curves were linear in the ranges measured: 12.5-1000 ng mL^{-1} for most fragrances and 2500-10000 ng mL^{-1} for α-asarone and myristicin, respectively. To evaluate the reproducibility of our results, six measurements using the same chromatographic parameters were made of the same fragrances. The relative standard



Scheme 2: Diagram of horizontal diffusion cell *in vitro* skin permeation.

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Fragrances	MeOH-H_2O	MeOH-CH_3CN-H_2O (*v/v/v*)			
	70:30(v/v)	10:50:40	20:40:40	30:30:40	40:40:20
Cinnamyl alcohol	5.77	4.40	4.05	5.57	—[c]
Eugenol	8.80	6.51	7.38	8.37	3.44
Isoeugenol	9.49	6.85	7.89	8.98	4.13
Eugenol methyl ether	13.92	9.73	11.31	12.94	5.01
α-Asarone	15.43	10.56	12.48	14.38	5.15
Myristicin	18.87	12.56	14.96	17.20	5.55
Sarfole	19.73	13.95	16.72	19.37	6.05
4-Allylanisole	22.45	15.47	18.37	21.11	6.40
Trans-anethole	27.37	16.64	19.92	23.01	6.72

[c]Not determined

Table 1: Effect of different mobile phase on the retention time (min) of fragrances on a 250 x 4.6 mm Phenomnex C_{18} column.

deviation values were between 0.05% and 0.79%. The correlation coefficient ranged between 0.9990 and 0.9999.

Application to essential oils

The proposed LC-FD procedure was used to analyze the allergens of fragrances in essential oils. The primary purpose was to find allergens in these samples. With the aid of authentic reference samples, the amounts of these nine compounds in the essential oils were determined. The identity of the registered peaks was confirmed first by comparing the observed retention times of the peaks with those of the individual standard solutions. Figure 2 shows the HPLC of allergens from commercial cinnamon essential oil samples chromatographed using acetonitrile-methanol-water (30:30:40, *v/v/v*, pH 6.02). Analytical results essential oil of 11 from commercial products are given in Table 2. Clove bud, basil, cinnamon, and aniseed may be used to permit the permeation of essential oil creams into the skin since the amounts of eugenol, eugenol methyl ether, and anethole were higher for these than for the other essential oils.

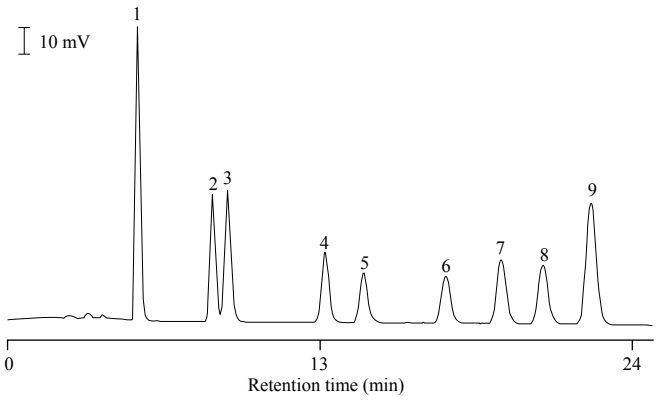

Figure 1: Separation of fragrances standards. A Phenomnex C_{18} analytical column (particle size 5 µm, 4.6×250 mm i.d.) eluted with acetonitrile-methanol-water (30:30:40, *v/v/v*, pH 6.02) containing 1.0 mM of phosphoric acid, flow rate 1.0 mL min^{-1}, Peak identification: (1) cinnamyl alcohol (250 ng mL^{-1}); (2) eugenol (250 ng mL^{-1}); (3) isoeugenol (250 ng mL^{-1}); (4) eugenol methyl ether (250 ng mL^{-1});(5) µ-asarone (2.5 µg mL^{-1}); (6) myristicin (2.5 µg mL^{-1}); (7) safrole (250 ng mL^{-1}); (8) 4-allylanisole (250 ng mL^{-1}); (9) anethole (250 ng mL^{-1}).

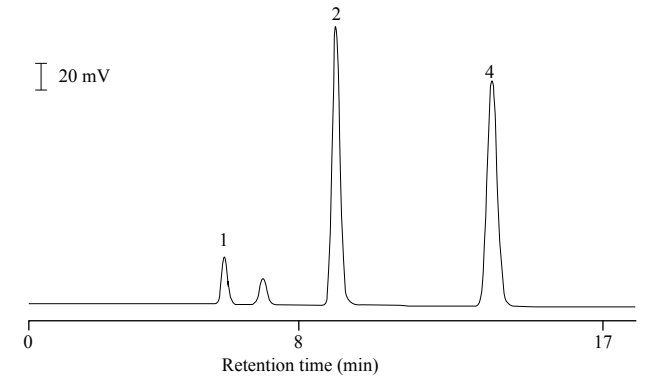

Figure 2: LC-FD chromatograms were obtained from commercial cinnamon essential oil. Peak: (1) cinnamyl alcohol; (2) eugenol; (4) eugenol methyl ether. Analysis conditions are identical to those listed in Figure 1.

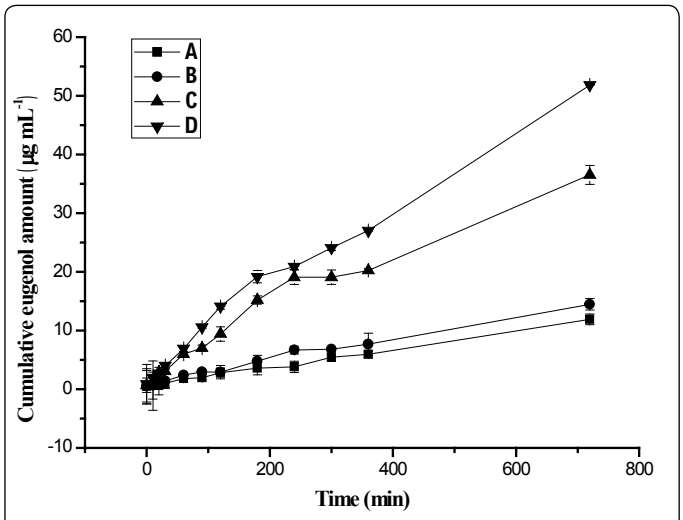

Figure 3: Effect of clove essential oil and Tween 80 on the skin permeation of eugenol in four preparations A-D; (A) without Tween 80 surfactant; (B,C and D) with various amount of Tween 80 surfactants. Each point represents average ± standard deviation(N=3, number of determination).

Application to cultured epidermal autograft skin

Clove essential oil increased the permeation of eugenol compared with those without the Tween 80. From B, C, and D preparations, the amount of eugenol that penetrated the skin increased as the dose of clove essential oil increased (Figure 3). Tween 80 increased the percutaneous absorption of eugenol. The permeability of a drug is influenced by its physicochemical properties and by the vehicle. Based on the *in vitro* results, the release concentrations of fragrances for different essential oils—clove bud, cinnamon, basil, and aniseed—increased with time (Figure 4 and Figure 5).The concentration of eugenol in clove bud was higher than in the other essential oils because clove contains between 39% and 92% eugenol, which is released more quickly and voluminously. Nevertheless, the release rates of cinnamon and basil increased slowly and were not significantly different between 420 and 720 min. From Table 2, three fragrances, *i.e.* cinnamyl alcohol (0.28%), eugenol (3.77%) and eugenol methyl ether (14.29%), exists in cinnamon essential oil. According to Figure 5, the release amounts increase with time indicating that

Retention No.	Compounds	Concentration(% , w/w)n=3[a]										
		Cl	T	B	Ci	A	G	C	R	J	L	Y
1.	Cinnamyl alcohol	–[c]	–	0.05 (3.20%)[b]	0.28 (4.20%)	–	–	–	–	–	–	0.01 (2.12%)
2.	Eugenol	92.73 (0.78%)	–	5.63 (2.52%)	3.77 (0.94%)	–	0.75 (7.03%)	0.34 (3.58%)	–	–	–	0.001 (1.15%)
3.	Isoeugenol	–	–	–	–	–	–	–	0.11 (2.33%)	1.33 (2.16%)	0.03 (3.37%)	0.14 (3.89%)
4.	Eugenol methyl ether	2.16 (0.21%)	–	0.22 (5.80%)	14.29 (2.27%)	–	–	–	–	0.40 (2..66%)	–	2.75 (3.42%)
5.	α-Asarone	–	0.05 (2.46%)	–	–	–	–	–	–	–	–	0.60 (3.43%)
6.	Myristicin	–	–	–	–	–	–	–	–	–	–	–
7.	Sarfole	–	–	–	–	–	–	–	–	–	–	–
8.	4-Allylanisole	–	–	0.78 (0.38%)	–	0.06 (1.66%)	–	–	–	–	–	–
9.	Trans-Anethole	–	–	–	–	86.62 (0.55%)	–	–	–	–	–	0.05 (2.80%)

[a]Number of determination [b]Coefficient of variation [c]Not determined

Table 2: Fragrance allylbenzenes of the essentials of clove bud (Cl), thyme white (T), basil (B), cinnamon (Ci), aniseed (A), geranium(G), clary sage(C), rosemary(R), jasminum(J), lavender(L), and ylang(Y) using RP- HPLC with fluorometric detection.

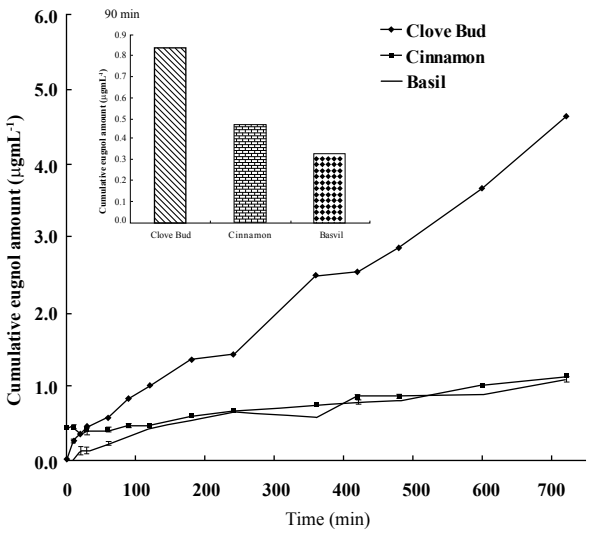

Figure 4: Cumulative amount of eugenol from clove bud, cinnamon, and basil essential oil micro-emulsions on the permeation through cultured epidermal autograft skin. Each point represents average ± standard deviation(N=3, number of determination).

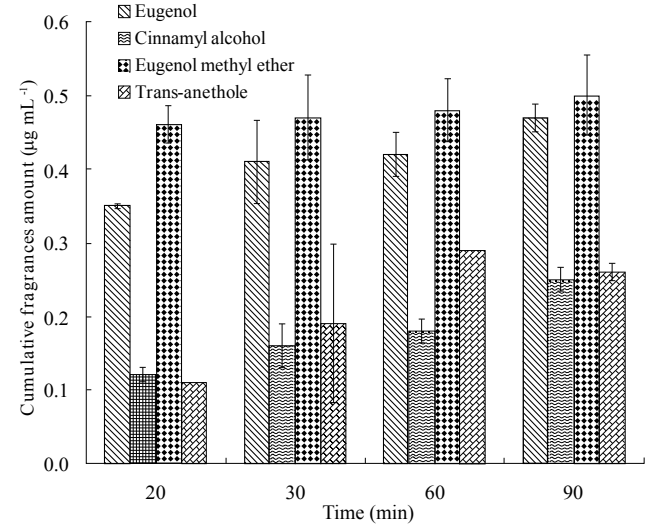

Figure 5: Cumulative amount of eugenol, cinnamyl alcohol, and eugenol methyl ether from cinnamon; and trans-anethole from aniseed essential oil micro-emulsions on the permeation through cultured epidermal autograft skin. Each point represents average ± standard deviation(N=3, number of determination)

membrane permeations are taking place as time progresses. From the data shows that *in vitro* skin permeation, the eugenol methyl ether concentration in cinnamon essential oil is higher than the others.

Conclusions

This LC-FD assay for simultaneously determining the allergens in the fragrances in essential oils and in cultured epidermal autograft skin has a low detection than other analysis doses. In the essential oils from 11 commercial products, we identified 8 different allylbenzenes (eugenol, isoeugenol, eugenol methyl ether, α-asarone, myristicin, safrole, 4-allylanisole, and trans-anethole) and 1 sensitizing fragrance. We conclude that essential oils are efficacious carriers for the transdermal delivery of fragrances because they allow the fragrances to penetrate the skin.

Acknowledgments

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