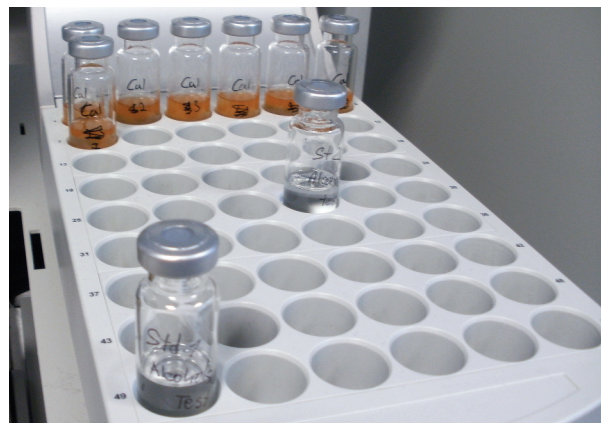
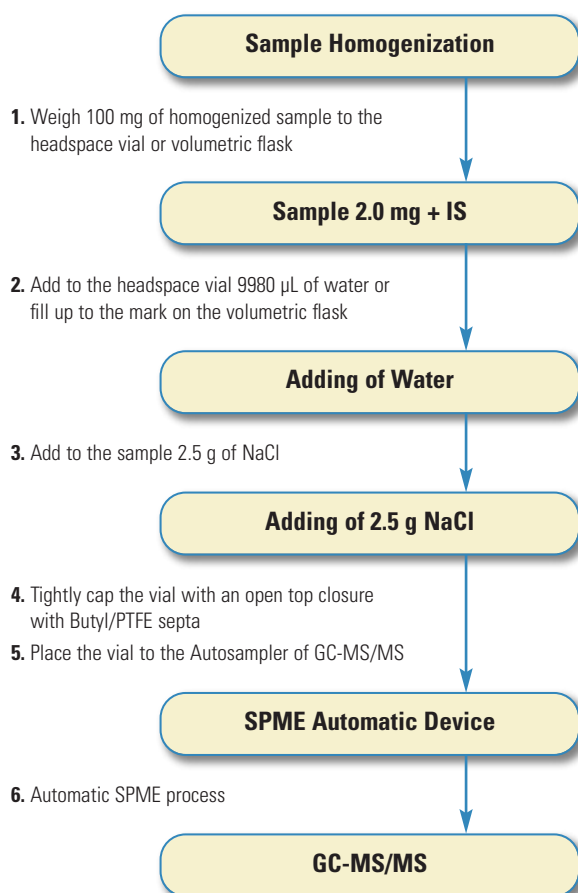


A Solid-phase Micro-extraction GC-MS/MS Method for Rapid Quantitative Analysis of Food and Beverages for the Presence of Restricted Biologically Active Flavorings¹

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1. Schematic of Method



range of physical and chemical composition such as liquids (spirit drinks and non-alcoholic beverages), semi-solid foods (soups, sauces and desserts) as well as solid foods (confectionery, chewing gum, fish, meat, bakery products and breakfast cereals). Without methods that can be routinely applied by the food industry, it is very difficult to control final levels of these flavoring substances in finished products, especially due to their high variability in levels between different plant species.

Headspace analysis is a very attractive methodology for analyzing volatiles, because it requires minimal sample preparation and can be automated. Of the headspace methods, solid-phase micro-extraction (SPME) is now probably the most widely used method in food analysis, offering many benefits over other headspace techniques.⁴ Although SPME is very well established for the analysis of flavorings, published methods have focused on individual food classes and no publications have truly tackled the issue of providing methodology for enforcement of regulations to control biologically active flavoring principles.

This publication describes a SPME method, utilizing a generic approach based on three categories of food types which has been optimized for the simultaneous determination of seven volatile flavoring substances whose levels are controlled in EU² in specified foods.

3. Scope

This method can be applied to alcoholic and non-alcoholic beverages, semi-solid processed foods and solid foodstuffs to detect and quantify the presence of seven biologically active flavoring substances (coumarin, β -asarone, estragole, menthofuran, methyl eugenol, pulegone and thujone) at levels ranging from 0.5 to 3000 mg/kg.

2. Introduction

Despite a history of use in foods and beverages, some plant extracts are now regulated in the EU² and some, such as safrole, are banned from direct addition to foods in the U.S.³

European Regulation 1334/2008² stipulates that 15 flavoring substances are banned from direct addition to foods or beverages in their chemically pure form. These flavoring compounds are agaric acid, aloin, capsaicin, coumarin, hypericine, β -asarone, estragole, hydrocyanic acid, menthofuran, methyleugenol, pulegone, quassin, safrole, teucrin A and α and β -thujone. Ten of these substances are permitted in food and beverages at stipulated levels, but only when they are naturally present in flavorings and food ingredients.² The stipulated foodstuffs cover a wide

Key Words

- TSQ Quantum XLS
- Beverages
- Biologically Active Flavorings
- Semi-Solid Food
- Solid Food
- Solid-phase Micro-extraction

4. Principle

The method employs automated headspace solid-phase micro-extraction (HS/SPME) for extraction of the targeted compounds (biologically active flavorings) from very broad types of matrices using a polydimethylsiloxane (PDMS) SPME fiber. Samples are placed in the headspace vials, fortified with labeled internal standards and water along with sodium chloride (NaCl) is added. Headspace vials are tightly sealed and after achieving equilibration headspace partition, the headspace is sampled automatically and analyzed by simultaneous GC-MS/MS using a Thermo Scientific TSQ Quantum XLS gas chromatography triple quadrupole mass spectrometer system.

5. Reagent List

	<i>Fisher Scientific Part Number</i>
5.1 Purified Water (obtained from Thermo Scientific Barnstead EASYpure II water system)	3125753
5.2 Sodium Chloride (extra pure)	194090010
5.3 Saccharose (extra pure)	S/8560/53
5.4 Ethanol (purity 99.9%)	E/0665DF/17
5.5 Methanol (purity 99.9%)	M/4058/17

6. Calibration Standards

6.1 Biologically Active Flavorings

- 6.1.1 Beta-asarone – purity 72% (Dr. Ehrenstorfer)
- 6.1.2 Coumarin (1,2-benzopyrone) – purity 99.5% (Dr. Ehrenstorfer)
- 6.1.3 Estragole (1-Allyl-4-methoxybenzene) – purity \geq 98.5% (Sigma-Aldrich)
- 6.1.4 Menthofuran – purity \geq 99% (Sigma-Aldrich)
- 6.1.5 Methyleugenol (4-Allyl-1,2-dimethoxybenzene) – purity 99.5% (Sigma-Aldrich)
- 6.1.6 Pulegone – purity 98.8% (Sigma-Aldrich)
- 6.1.7 Thujone (alpha and beta) – purity \geq 99% (Sigma-Aldrich)

6.2 Internal Standards

- 6.2.1 Coumarin – 5, 6, 7, 8 – D4, c = 100 μ g/mL in acetone (Dr. Ehrenstorfer)
- 6.2.2 Dicyclohexylmethanol – purity 98% (Sigma-Aldrich)

7. Standards Preparation

- 7.1 Stock standard solutions of flavorings (1000 μ g/mL): Weigh 25.00 mg of the compound (recalculate the amount regarding actual purity of the standard) into volumetric flasks, dissolve in methanol and dilute to 25 mL. Solutions can be stored at 4 °C for at least three months.
- 7.2 Working standard solution of 7 flavorings (1 respectively 10 μ g/mL for coumarin): Transfer 25 μ L of stock solution of thujone, menthofuran, estragole, pulegone, methyl eugenol and β -asarone (1000 μ g/mL) and 250 μ L of stock solution of coumarin (1000 μ g/mL) to a 25 mL volumetric flask and dilute to marked volume with water. Solution should be prepared fresh every time before using.
- 7.3 Stock standard solution of internal standard dicyclohexylmethanol (1000 μ g/mL): Weigh 25.00 mg of the compound (recalculate the amount regarding actual purity of the standard) into a volumetric flask, dissolve in methanol and dilute to 25 mL. Solution can be stored at 4 °C for at least three months.
- 7.4 Working standard solution of internal standard dicyclohexylmethanol (10 μ g/mL): Transfer 100 μ L of stock solution of dicyclohexylmethanol (1000 μ g/mL) to a 10 mL volumetric flask and dilute to marked volume with water. Solution should be prepared fresh every time before using.

8. Apparatus

	<i>Fisher Scientific Part Number</i>
8.1 High speed blender – ULTRA-TURRAX®	3565000
8.2 ULTRA-TURRAX – Dispergation tool	1713300
8.3 ULTRA-TURRAX – Plug-in coupling	1024200
8.4 Waring laboratory blender	68909
8.5 Fisher precision balance	XP-1500FR
8.6 Sartorius analytical balance	ME235S
8.7 SPME holder – TriPlus™ SPME Kit	190.504.34
8.8 TSQ Quantum XLS™ Triple Quadrupole Mass Spectrometer – Thermo Fisher Scientific (Austin, TX USA)	
8.9 Thermo Scientific TRACE GC Ultra system with automated SPME system – Thermo Fisher Scientific (Austin, TX USA)	

9. Consumables

Fisher Scientific
Part Number

9.1	GC column – TraceGOLD TG-5MS 5% diphenyl and 95% dimethyl polysiloxane stationary phase, 30 m, 0.25 mm ID, 0.25 µm film thickness (Thermo Fisher Scientific, Bellefonte, PA USA)	26098-1420
9.2	SPME fiber – coated with PDMS, d _f 100 µm	57341-U <i>Supelco Bellefonte, PA USA</i>
9.3	Headspace vials – 20 mL flat bottom, clear glass, beveled edge	3205551
9.4	PTFE – faced butyl rubber septa for headspace vials – 20 mm, septa Butyl/PTFE	3205532
9.5	Capping device – Manual Crimper for 20 mm Aluminum Crimp Seals	C4020-100
9.6	Pipette Finnpiquette 100–1000 µL	3214535
9.7	Pipette Finnpiquette 20–200 µL	3214534
9.8	Pipette Finnpiquette 10–100 µL	3166472
9.9	Pipette Finnpiquette 500–5000 µL	3166473
9.10	Pipette Finnpiquette 1000–10000 µL	3214536
9.11	Pipette holder	3651211
9.12	Pipette tips 0.5–250 µL, 500/box	3270399
9.13	Pipette tips 1–5 mL, 75/box	3270420
9.14	Pipette tips 100–1000 µL, 200/box	3270410
9.15	Pipette tips 20000–10000 µL, 40/box	3270425
9.16	Pipette Pasteur – soda lime glass 150 mm	FB50251
9.17	Pipette suction device	3120891
9.18	Spatula, 18/10 steel	3458179F
9.19	Spatula, nylon	3047217
9.20	Wash bottle, PTFE	3149330
Glassware		
9.21	Beaker, 50 mL	965 32 10
9.22	Beaker, 100 mL	965 32 20
9.23	Volumetric flask, 10 mL	FB50143
9.24	Volumetric flask, 25 mL	FB50147

10. Procedure

Preparation of the Instrument – Before starting to work with the instrument or preparation it for work in SPME mode, please read carefully the relevant chapter of the Thermo Scientific TriPlus Operating Manual and Section IV in the Thermo Scientific TriPlus Standard Operating Procedures. There is described all necessary maintenance during installation of the SPME holder and SPME fiber.

10.1 Sample Preparation

Solid and Semi-solid matrices

10.1.1 Homogenize 150 g of sample in a high-speed blender (soups, sauces and pesto) or in a Waring laboratory blender (solid matrices like muesli) for 5 min, and then accurately weigh 0.1 g directly into a headspace vial.

10.1.2 Add 10 µL of working standard solution of dicyclohexylmethanol, 10 µL of standard solution of coumarin-d₄, and add 9980 µL of water using micropipettes of appropriate sizes.

Liquid Matrix

10.1.3 Weigh 0.1 g directly into a headspace vial add 10 µL of working standard solution of dicyclohexylmethanol, 10 µL of standard solution of coumarin-d₄ and add 9980 µL of water using micropipettes of appropriate sizes.

10.1.4 In both cases, add 2.5 g NaCl, seal with a PTFE-faced butyl rubber septum and cap the sample with the crimping device.

10.1.5 For calibration purposes, use blank foodstuffs representative of each of the respective matrix types.

- Liquid matrix (mainly representing alcoholic drinks) comprising a 40% solution of aqueous ethanol used as blank material
- Semi-solid matrix (mainly representing sauces and pesto) comprising pure tomato sauce used as blank material
- Solid matrix (mainly representing muesli) comprising oat flakes used as blank material

For solid and semi-solid matrices, use the volumes of standards and internal standards as shown in Table 1, and for liquid matrices use corresponding volumes as shown in Table 2.

10.2 Automated SPME Analysis

- 10.2.1 Use the fiber coated with polydimethylsiloxane 100 μm (PDMS-100) and condition the fiber before use by insertion into the GC injector as recommended by the manufacturer.
- 10.2.2 Load the SPME autosampler with headspace vials containing the prepared samples (up to a maximum of 54 vials per tray).
- 10.2.3 Commence the SPME program which consists of swirling the vial for 5 min at 50 $^{\circ}\text{C}$, then inserting the fiber into the head-space for 40 min at 50 $^{\circ}\text{C}$ as the solution is swirled again, then transferring the fiber to the injector for desorption at 250 $^{\circ}\text{C}$ for 5 min. At the end of the program, the fiber is transferred to the second injector (instead of the conditioning station) for cleaning and conditioning at 250 $^{\circ}\text{C}$ for 5 min.

10.3 GC Analysis

GC analysis is performed on a TRACE GC Ultra™ system with automated SPME system (Thermo Fisher Scientific, Austin, TX USA). The GC conditions were as follows:

Column: TraceGOLD TG-5MS 5% diphenyl and 95% dimethyl polysiloxane stationary phase (30 m, 0.25 mm ID, 0.25 μm film thickness)

Injection mode: splitless

Injection port temperature: 250 $^{\circ}\text{C}$

Left carrier flow: 1.2 mL/min

Split flow: 50 mL/min

Splitless time: 3 min

Conditioning injector temperature: 250 $^{\circ}\text{C}$

Right carrier flow: 0.1 mL/min

Transfer line temperature: 250 $^{\circ}\text{C}$

Oven temperature: 60 $^{\circ}\text{C}$ hold for 1 min; to 120 $^{\circ}\text{C}$ with 15 $^{\circ}\text{C}/\text{min}$; hold for 2 min; to 225 $^{\circ}\text{C}$ with 30 $^{\circ}\text{C}/\text{min}$; hold for 1 min; to 280 $^{\circ}\text{C}$ at 30 $^{\circ}\text{C}/\text{min}$, hold for 10 min

10.4 Tandem MS/MS Detection

MS analysis is carried out using a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Fisher Scientific, Austin, TX USA).

Ionization mode: electron impact (EI) positive ion at 70 eV ionization energy

Emission current: 30 μA

Ion source temperature: 250 $^{\circ}\text{C}$

Scan type: selected reaction monitoring (SRM)

Cycle time: 0.1 s

Peak width: Q1/Q3 the full width of a peak at half its maximum height (FWHM) of 0.70 Da

Collision gas (Ar) pressure: 1.0 mTorr

The parameters for selected reaction monitoring (SRM) analysis for targeted compounds and internal standards are displayed in the Table 3.

11. Calculations of Results

11.1 Identification

It is confirmed by the presence of transition ions (quantifier and qualifier) at retention times ($\pm 0.05\%$) to the corresponding standards. In multiple reaction monitoring (MRM) mode the measured peak area ratios for qualifier to quantifier ion should be in close agreement ($\pm 20\%$) with those of the standards as shown in Table 3. The quantifier and qualifier ion were selected among the product ions produced by the fragmentation of the selected parent ion on the basis of the intensity.

11.2 Quantification

It employs internal standardization using peak area ratios for standards in matched matrices. Dicyclohexylmethanol is used as the internal standard for the six flavor compounds (thujone, menthofuran, estragole, pulegone, methyl eugenol and β -asarone), and coumarin- d_4 is used as internal standard for coumarin. Plot the calibration curves as the relative peak areas (analyte versus the corresponding internal standard) as a function of the compound concentration. The flavoring concentration (c_{fi}) in the samples is determined from the equation:

$$c_{FI} = \left(\frac{A_{FI}}{A_{IS}} \right) - b/a$$

where,

c_{FI} – flavoring concentration in mg/kg

A_{FI} – peak area of the flavoring

A_{IS} – peak area of internal standard

b – the y-intercept

a – the slope of calibration curve

Samples initially found to contain levels of flavoring substances outside the linear range need to be appropriately diluted, and the dilution factor taken into account in the final calculations.

12. Method Validation

Validation was carried out in terms of specificity, linearity, precision, limit of detection (LOD) and quantification (LOQ), accuracy and robustness. Finally, the applicability of the method to the determination of targeted flavorings in a number of commercial samples was demonstrated.

The method performance was established by spiking experiments with blank matrices (solid – oat flakes; semi-solid – pure tomato sauce; and liquid – water with ethanol) with a mixture of targeted compounds.

12.1 Specificity

Using Selected Reaction Monitoring (SRM) the specificity is confirmed based on the presence of the transition ions (quantifier and qualifier) at the correct retention times corresponding to those of the respective flavoring standards. The measured peak area ratios of qualifier/quantifier ion have to be in close accordance with the ion ratios of the standards as indicated in Table 3.

12.2 Linearity and Calibration Curve

The linearity of calibration curves is assessed over the range from 0.01–2.0 mg/kg (for six flavorings) and 0.1–2.0 mg/kg for coumarin. In all cases, the correlation coefficients of linear functions has to be > 0.99. The calibration curves are created from seven matrix-matched calibration standards which are injected in each batch in duplicate.

12.3 Precision

The relative standard deviation (% RSD) was determined by injecting six replicates of spiked samples of three different matrices at two different levels. For the liquid matrix, aqueous ethanol (40%) was used as the blank matrix (with the addition of various amounts of saccharose to simulate liqueurs and energy drinks), for semi-solid matrices pure tomato sauce was used, and for the solid matrix oat flakes. The samples were spiked at 0.1 and 1 mg/kg levels and six replicate analyses were analyzed. For six flavorings (β -asarone, estragole, menthofuran, methyl eugenol, pulegone and thujone) the first level of addition was 0.1 mg/kg, and for coumarin the addition was at 1 mg/kg. The second level of addition for six flavorings was 1 mg/kg, again with coumarin being at a higher level of 10 mg/kg. The results that establish method precision are shown in Table 4, indicating RSDs from 2 to 21%. All precisions are acceptable for a regulatory method, with liquid and semi-solid foods offering a better performance than solid foods.

12.4 Limits of Detection (LOD) and Quantification (LOQ)

Limits of detection and quantification were estimated following the IUPAC approach which consisted of analyzing the blank sample to establish noise levels and then estimating LODs and LOQs for signal/noise, 3 and 10 respectively. The values for three matrices (solid, semi-solid and liquid) are shown in Table 5 and, in all cases, these values far exceed requirements to test for compliance to regulatory limits in which 0.5 mg/kg is the lowest level which is controlled.

12.5 Accuracy

Accuracy was evaluated by comparing found values with spikes by standard addition. The optimization method was used to analyze three types of matrix. For the liquid matrix, spiking was into 40% aqueous ethanol, for a semi-solid matrix pure tomato sauce was used, and for a solid matrix oat flakes were used. The samples were spiked at levels of 0.1 and 1 mg/kg in six replicates. For six flavorings (β -asarone, estragole, menthofuran, methyl eugenol, pulegone and thujone) at 0.1 mg/kg and for coumarin at 1 mg/kg for level 1, and six flavorings at 1 mg/kg and 10 mg/kg for coumarin for level 2. The results in Table 6 show good accuracy, except in the case of solid matrices for which overestimations are indicated.

13. Conclusion

This single laboratory validated method is capable of determining levels of any one of seven biologically active flavoring substances which have use restrictions in composite foodstuffs. The method can cover all food types based on a generic approach of selecting the category of either a liquid, semi-solid or solid matrix, and then following the optimized conditions for that category. The method has a sensitivity which far exceeds regulatory requirements and the use of MS/MS for detection guarantees a high level of confidence in correct identification based on ion ratios. We recommend this method for use for enforcement of limits of biologically active flavorings in foods.

14. References

1. For details of this research please see: Bousova K., Mittendorf K., Paez V., Senyuva H., A Solid-Phase Micro-Extraction GC-MS/MS Method for Rapid Quantitative Analysis of Food and Beverages for the Presence of Legally Restricted Biologically Active Flavorings. *J. AOAC*. Accepted for publication, 2011.
2. Regulation (EC) No 1334/2008 of 16 December 2008 on flavorings and certain food ingredients with flavoring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. *Official Journal of the European Union*. (2008) L 354/34-50.
3. Code of Federal Regulations Title 21CFR189.180 [Revised as of April 1, 2010]
4. Kataoka, H., Lord, H. L., & Pawliszyn, J. (2000). *J. Chromatogr.*, A, 880, 35-62.

Concentrations of 6 Flavoring Standards + Coumarin (mg/kg)	Concentration of DCHM & coumarin-d ₄ Standard (mg/kg)	Volume of Working Standard Solution Added (μL)	Volume of Working Internal Standard DCHM Added (μL)	Volume of Standard Solution of coumarin-d ₄ Added (μL)	Volume of Water Added (μL)
0.01 and 0.1	1 and 10	1	10	10	9979
0.05 and 0.5	1 and 10	5	10	10	9975
0.1 and 1	1 and 10	10	10	10	9970
0.5 and 5	1 and 10	50	10	10	9930
1 and 10	1 and 10	100	10	10	9880
1.5 and 15	1 and 10	150	10	10	9830
2 and 20	1 and 10	200	10	10	9780

DCHM = dicyclohexylmethanol internal standard

Table 1: Preparation of matrix matched standards for semi-solid and solid matrices

Concentrations of 6 Flavoring Standards and Coumarin (mg/kg)	Concentration of DCHM & coumarin-d ₄ Standard (mg/kg)	Volume of Working Standard Solution Added (μL)	Volume of Working Internal Standard DCHM Added (μL)	Volume of Standard Solution of coumarin-d ₄ Added (μL)
0.01 and 0.1	1 and 10	1	10	10
0.05 and 0.5	1 and 10	5	10	10
0.1 and 1	1 and 10	10	10	10
0.5 and 5	1 and 10	50	10	10
1 and 10	1 and 10	100	10	10
1.5 and 15	1 and 10	150	10	10
2 and 20	1 and 10	200	10	10

DCHM = dicyclohexylmethanol internal standard

Table 2: Preparation of matrix matched standards for liquid samples

Flavoring Substance	Retention Time (min)	Molecular Weight	Precursor Ion	Quantifier Ion	Qualifier Ion 1	Ion Ratio Qual/Quant	Collision Energy (V)
Thujone	5.86	152.23	110.03	95.02	67.05	0.20	10
Menthofuran	6.66	150.22	107.94	79.01	77.00	0.52	15
Estragole	7.16	148.20	147.98	91.06	115.10	0.82	25
Pulegone	7.74	152.23	152.01	81.03	137.04	0.44	10
Methyl eugenol	9.18	178.23	177.98	147.03	163.05	0.72	15
Coumarin	9.50	146.14	145.92	117.99	89.93	0.68	20
coumarin-d ₄	9.49	150.17	149.92	122.02	93.98	0.14	15
Dicyclohexylmethanol	10.42	196.33	112.27	79.05	81.05	0.90	10
β-Asarone	10.43	208.26	207.99	165.08	193.11	0.91	15

Table 3: GC-MS/MS parameters for selected reaction monitoring of flavorings

Analyte	Relative Standard Deviation (RSD %)							
	Level 1 mg/kg	Level 2 mg/kg	Liquid Matrix		Semi-solid Matrix		Solid Matrix	
			Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
Thujone	0.1	1	6	5	8	5	17	17
Menthofuran	0.1	1	14	5	2	15	19	21
Estragole	0.1	1	6	4	9	8	17	13
Pulegone	0.1	1	3	6	7	5	8	16
Methyl eugenol	0.1	1	7	6	3	4	9	12
Coumarin	1	10	13	2	13	3	13	7
β-Asarone	0.1	1	8	11	6	5	4	10

Table 4: RSD (%) of 6 spiked samples at 2 levels

Analyte	Liquid Matrix		Semi-solid Matrix		Solid Matrix	
	LOD (mg/kg)	LOQ (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)	LOQ (mg/kg)	LOQ (mg/kg)
Thujone	0.001	0.005	0.01	0.05	0.003	0.01
Menthofuran	0.001	0.005	0.003	0.01	0.005	0.01
Estragole	0.005	0.01	0.001	0.005	0.005	0.01
Pulegone	0.001	0.005	0.003	0.01	0.001	0.005
Methyleugenol	0.001	0.005	0.01	0.05	0.01	0.05
Coumarin	0.1	0.5	0.1	0.5	0.1	0.5
β -Asarone	0.001	0.005	0.01	0.05	0.003	0.01

Table 5: Limits of detection and quantification (LODs and LOQs)

Analyte	Recoveries (%)							
	Level 1 mg/kg	Level 2 mg/kg	Liquid Matrix		Semi-solid Matrix		Solid Matrix	
			Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
Thujone	0.1	1.0	95	99	83	121	146	131
Menthofuran	0.1	1.0	121	83	50	83	126	124
Estragole	0.1	1.0	115	90	129	125	123	117
Pulegone	0.1	1.0	107	88	98	105	119	127
Methyl eugenol	0.1	1.0	99	91	106	102	124	113
Coumarin	1.0	10.0	96	97	96	111	107	111
β -Asarone	0.1	1.0	85	121	62	90	115	116

Table 6: Recoveries (%) for spiked samples at 2 levels

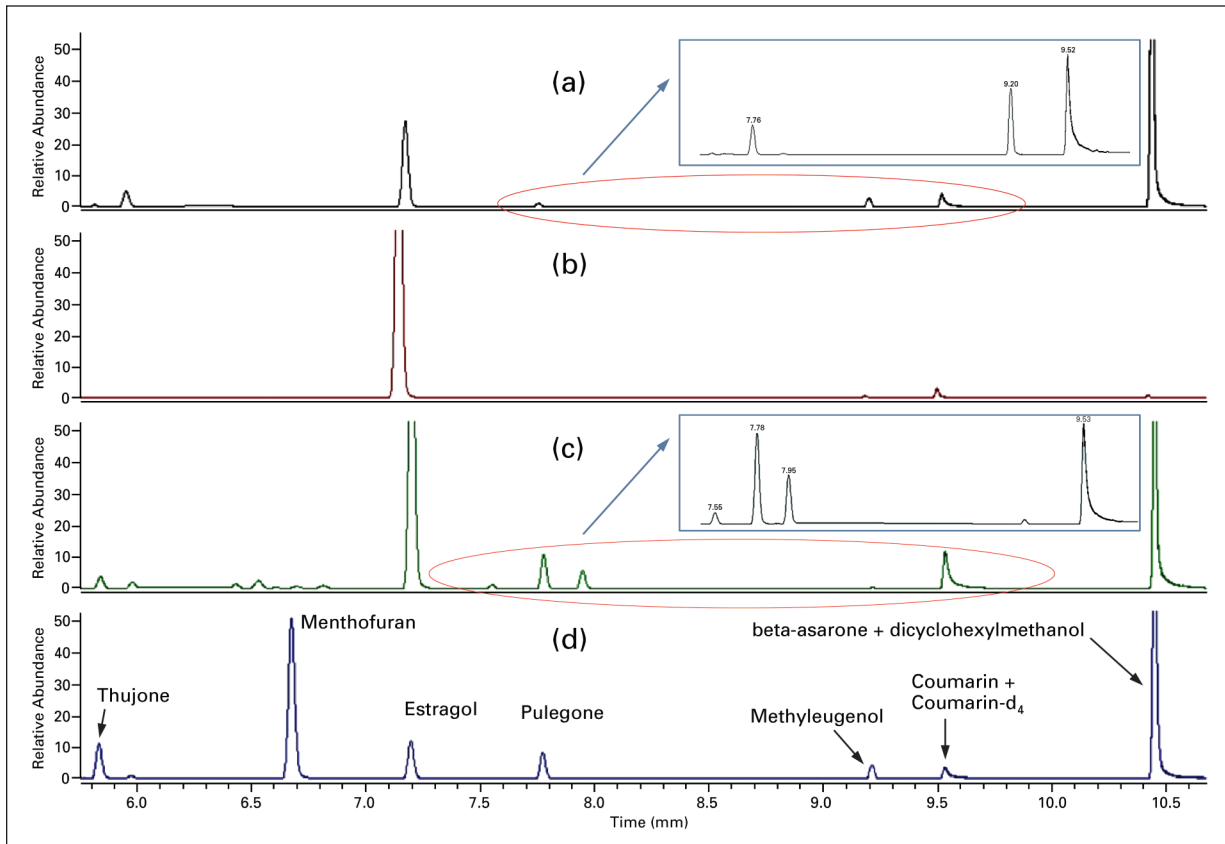


Figure 1: MRM chromatograms for (a) Herbal liqueur containing estragole, pulegone & methyl eugenol; (b) Pesto sauce containing estragole and methyl eugenol; (c) Herbal tea containing thujone, menthofuran, estragole, pulegone, methyl eugenol and coumarin; (d) mixture of seven flavoring standards and two internal standards

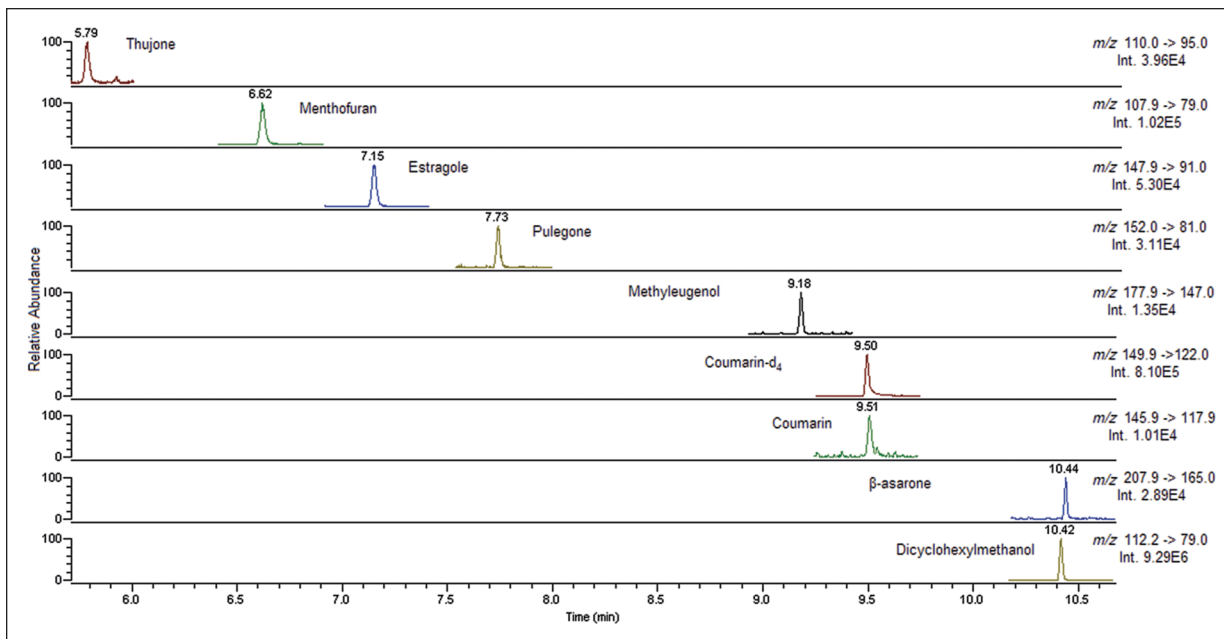


Figure 2: Chromatogram of the matrix matched standard for semi-solid matrixes (pure tomato sauce used as blank material) with concentration 0.01 mg/kg for β-asarone, estragole, menthofuran, methyleugenol, pulegone and thujone; 0.1 mg/kg for coumarin and for internal standards 1 mg/kg for dicyclohexylmethanol and 10 mg/kg for coumarin-d₄. The figure shows SRM traces for 7 flavoring substances plus 2 internal standards.

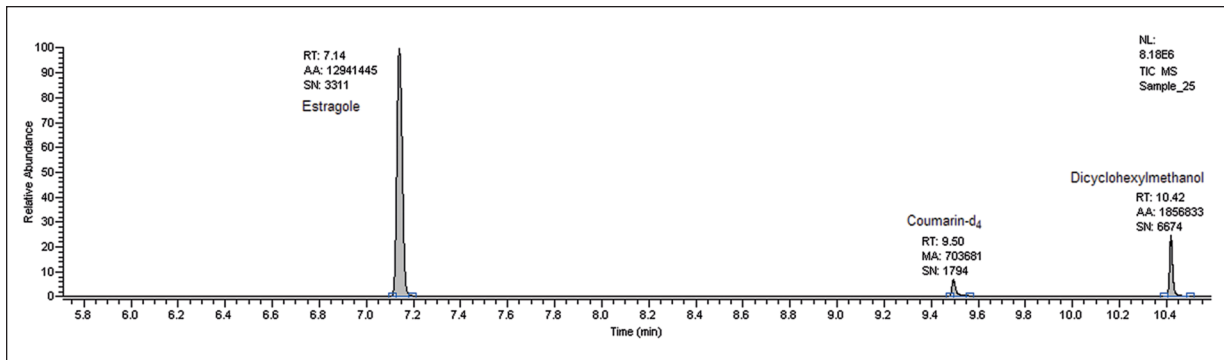


Figure 3: Chromatogram of basil tomato sauce; detected flavoring substance: estragole – 3.54 mg/kg; internal standards: coumarin-d₄ – 10 mg/kg and dicyclohexylmethanol – 1 mg/kg

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