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Analytical Methods

A straightforward method to determine flavouring substances in food by GC–MS

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ABSTRACT

A straightforward GC–MS method was developed to determine the occurrence of fourteen flavouring compounds in food. It was successfully validated for four generic types of food (liquids, semi-solids, dry solids and fatty solids) in terms of limit of quantification, linearity, selectivity, matrix effects, recovery (53–120%) and repeatability (3–22%).

The method was applied to a survey of 61 Dutch food products. The survey was designed to cover all the food commodities for which the EU Regulation 1334/2008 set maximum permitted levels. All samples were compliant with EU legislation. However, the levels of coumarin (0.6–63 mg/kg) may result in an exposure that, in case of children, would exceed the tolerable daily intake (TDI) of 0.1 mg/kg bw/day. In addition to coumarin, estragole, methyl-eugenol, (R)-(+)-pulegone and thujone were EU-regulated substances detected in thirty-one of the products.

The non-EU regulated alkenylbenzenes, *trans*-anethole and myristicin, were commonly present in beverages and in herbs-containing products.

1. Introduction

The incorporation of herbs and other plants in foods as flavouring agents has a long tradition, dating back to the origins of cooking. In fact, today's society is increasingly demanding food products prepared with natural ingredients. Although there is a widespread belief that "natural is good," some plants and plant extracts used for flavouring may contain substances, the carcinogenic or genotoxic effects of which have been demonstrated or are currently under study (CoE, 2005; EFSA, 2005b, 2008; van den Berg, Restani, Boersma, Delmulle, & Rietjens, 2011).

One of the categories of plant metabolites of concern is alkenylbenzenes. Two classes of alkenylbenzenes have been identified: allylbenzenes with a 2,3-double bond such as estragole, methyl eugenol, safrole, myristicin and elemicin, and propenylbenzenes with a 1,2-double bond such as *trans*-anethole and β-asarone. Estragole, safrole, methyl eugenol, *trans*-anethole, myristicin and elemicin are present in many herbs used as bases for flavourings, and in variable concentrations in spices such as fennel, anise, nutmeg, coriander, cinnamon, basil, ginger and black pepper (Avila, Zougagh, Escarpa, & Rios, 2009; Siano et al., 2003). Vermouths, bitters, and liquors prepared from *Acorus calamus* contain the biologically active β-asarone (Lee, Yu, Sim, Ko, & Hong, 2013). Coumarin is a flavouring substance belonging to the family of benzopyrones. Cinnamon is an important source of coumarin (Lungarini, Aureli, & Coni, 2008); and, therefore, often occurs with cinnamaldehyde. Both flavouring substances are present at relatively high concentrations in a wide variety of plants in some essential oils, specifically cassia leaf, cinnamon leaf, bark, lavender, and peppermint oils (Bousova, Mittendorf, & Senyuva, 2011). Pulegone (monoterpenoid cyclic ketone) is one of the main constituents of peppermint and pennyroyal oils but it also occurs at lower levels in other food commodities such as oregano, beans and tea. Pulegone can be detected in many mint products, such as candies and chewing gum (Siano, Catalfamo, Cautela, Servillo, & Castaldo, 2005). Many mint products also contain menthofuran, and the hepatotoxicity of pulegone is due to its biotransformation to menthofuran (Thomassen, Slattery, & Nelson, 1988). Thujones (α- and β-isomers) are the active compounds in absinthe, but they also naturally occur in a number of aromatic plants commonly used as food/beverage flavourings. Thujones are *gamma*-aminobutyric acid (GABA) antagonists and, while they are likely to produce muscle spasms when consumed in large doses, there is a lack of evidence for causing hallucinations or the disease called absinthism (Dawidowicz & Dybowski, 2012). Furocoumarins are a family of natural plant constituents with phototoxic and photomutagenic properties that are found mainly in vegetables and fruits such as

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celery, carrots, parsnips (Ostertag, Becker, Ammon, Bauer-Aymanns, & Schrenk, 2002) and citrus (Gorgus, Lohr, Raquet, Guth, & Schrenk, 2010). One of these furanocoumarins, 8-methoxypsoralen (8-MOP), has been classified by the International Agency for Research in Cancer (IARC) as carcinogenic to humans in class 1A (IARC, 1987). The phenol derivates thymol and carvacrol are the main constituents of the essential oils of thyme and oregano. Their antioxidant, antibacterial, antiviral, antifungal and antiparasital properties have been extensively reported (Lopez, Sanchez, Batlle, & Nerin, 2007). Carvacrol may be added to different food products such as baked goods, non-alcoholic beverages and chewing gums. Recently, some *in vivo* studies on rats have revealed high genotoxic effect for these two compounds at 10 mg/g (carvacrol) and 40 mg/g (thymol) (Azirak & Rencuzogullari, 2008).

Between 1999 and 2002, the Scientific Committee on Food (SCF) carried out a risk assessment for variety of these compounds (SCF, 2001a, 2001b, 2002a, 2002b, 2003). The European Food Safety Authority (EFSA) revised the risk assessment for coumarin (EFSA, 2008) and for pulegone and menthofuran (EFSA, 2005b), and has recently published an updated *compendium* of botanicals that have been reported to contain toxic, addictive, psychotropic or other substance of concern (EFSA, 2012). Among other sources, these opinions have resulted in the creation and update of the European legislation. Regulation EC 1334/2008 (EU, 2008) provides an approved list of flavouring and source materials for use in and on foods, as well as their conditions of use. Coumarin, β-asarone, menthofuran, methyleugenol, pulegone, safrole and thujone are forbidden to be added as such to food. They can only be present at certain levels in certain food commodities if they are naturally present as an ingredient in the flavouring substance or an ingredient with flavouring properties. The regulatory limits range from 0.5 mg/kg for α- and β-thujone in non-alcoholic beverages produced from *Artemisia* species, up to 3000 mg/kg for menthofuran for microbreath freshening confectionery. For methyl eugenol, estragole and safrole no limits are applicable when presented as fresh, dried or frozen herbs and spices. The chemical and physical composition of the foodstuffs covered by EU legislation is broad and is comprised of liquids, such as spirit drinks and non-alcoholic beverages; semi-solid foods, such as soups and sauces; dry-solid foods, such as bakery products, chewing gum or breakfast cereals; and greasy-solid foods, such as fish or meat products.

A review on the analytical methods for the detection of phytochemicals in plant materials and food has been recently published in 2011 (Zhao, Lv, Chen, & Li, 2011). Although various analytical approaches have been developed to determine individual flavourings in different food matrices, none of them was specifically for regulatory purposes or for being routinely used by the food industry. In fact, they only covered a limited number of the regulatory flavouring compounds. Siano developed a method to determine the content of estragole, methyl eugenol and safrole in foodstuff (Siano et al., 2003) and the content of pulegone in mint-flavoured products (Siano et al., 2005). The method comprised of a simultaneous distillation extraction (SDE), using a Likens–Nickerson apparatus and dichloromethane as extraction solvent, and gas chromatography–mass spectrometry (GC–MS). Although the method was fit for purpose in terms of accuracy, it was not very environmental-friendly and not suitable for routine analysis. Solvent extraction with methanol or ethanol aqueous mixtures and further analysis by liquid chromatography and diode array detection (HPLC–DAD) has been applied to determine coumarin and cinnamaldehyde in Italian foods (Lungarini et al., 2008) and coumarin in German products (Sproll, Ruge, Andlauer, Godelmann, & Lachenmeier, 2008). However, alcoholic solvents are not appropriate for the extraction of aldehydes, such as cinnamaldehyde, due to the aldol reaction between the –OH group and the aldehyde (Lv, Huang, Yang, Li, & Li, 2010). Besides, HPLC–DAD is not a very sensitive technique for the analysis of the other more volatile regulated flavouring substances. The problem of the sensitivity has been partially solved by using on-column preconcentration (Avila et al., 2009), with detection limits ranging 0.009–0.0015 mg/L for coumarin, methyl eugenol, pulegone, myristicin, anethole and estragole. The application of this method for routine purposes needs to be further explored. Walsh developed a method focussed only on thujone in spirit beverages that comprised of liquid–liquid extraction with 1,1,2-trichloro-1,2,2-trifluoroethane as extraction solvent and GC–MS analysis (Walch, Kuballa, Stuhlinger, & Lachenmeier, 2011). Cleaner sample extraction techniques, such as stir-bar-sorptive extraction (SBSE), have been validated and successfully applied for the determination of estragole and *trans*-anethole in fennel tea (Raffo, Nicoli, & Leclercq, 2011) and safrole, *trans*-cinnamaldehyde and myristicin in cola-flavoured soft drinks (Raffo, D'Aloise, Magri, & Leclercq, 2013). Nevertheless, SBSE is not widely implemented in routine and control laboratories.

Bousova et al. (2011) was the first to develop and validate a method intended for EU regulation enforcement. Their method, which included seven regulated flavouring substances, was based on headspace solid-phase microextraction (HS-SPME) and tandem mass spectrometry (MS/MS) to achieve the desired sensitivity. Although HS-SPME prevents co-extraction of less volatile matrix constituent, the analysis time involved (which included incubation, extraction (40 min) and instrument time) was relatively long. The effect of the matrix composition on the release of the analytes to the headspace was solved by dilution with water, the addition of salt and the use of small sample weights (0.1 g) in the headspace vial. Despite the fact that solid and semi-solid samples were homogenised before weighing, the sample size (0.1 g) might be difficult to ensure the representativeness of complex food matrices.

The aim of this work was to develop a method involving a fast and straightforward matrix-independent extraction procedure based on solvent extraction, and analysis using a relatively low cost GC–MS (single quadrupole) instrument. Solvent extraction is a simple, fast technique that does not require complex sample preparation equipment. Compared to headspace techniques, more matrix compounds are likely to be extracted, which may be disadvantageous with respect to selectivity and/or contamination of the GC system. However, since the regulatory limits are relatively high, solvent extraction was considered an attractive alternative to the previously described techniques. In addition to the regulated substances, a number of other flavouring substances, such as carvacrol, thymol, *trans*-anethole, 8-methoxypsoralen, myristicin, elemicin and *trans*-cinnamaldehyde, were included since they have been reported to be of concern by EFSA (EFSA, 2012). The method was applied during a survey of 61 samples of various types of food products collected from supermarkets in the Netherlands and Belgium.

2. Experimental

2.1. Chemicals and reagents

Ethyl acetate (EtOAc) and acetonitrile were purchased from Biosolve (Valkenswaard, the Netherlands). *n*-Hexane was supplied by Actu-all Chemicals (Oss, the Netherlands). Magnesium sulphate anhydrous and sodium chloride were purchased from Merck (VWR, Amsterdam, the Netherlands). Magnesium sulphate was dehydrated before use by heating at 400 °C overnight.

Certified standards of *trans*-anethole, estragole, (+)-menthofuran, thujone, methyl eugenol, safrole, myristicin, 8-methoxypsoralen (8-MOP), coumarin, (R)-(+)-pulegone, (S)-(−)-pulegone, thymol, carvacrol, *trans*-cinnamaldehyde and dicyclohexylmethanol were supplied by Sigma–Aldrich (Zwijndrecht, the Netherlands).

Elemicin was supplied by Synchem OHG (Felsberg/Altenburg, Germany) and β-asarone by Dr. Ehrenstorfer (Augsburg, Germany).

A stock standard solution of the internal standard (IS) dicyclohexylmethanol of 10,000 μg/g *n*-in hexane and a working solution of 1000 μg/g in ethyl acetate were prepared and kept at −20 °C.

Four mixed stock standard solutions of 10,000 μg/g containing a number of the target compounds were prepared in ethyl acetate and kept at −20 °C. From the mixed stock standard solutions, a working solution containing all the analytes at 1000 μg/g in ethyl acetate was prepared. From this working solution, two other working solutions of 100 μg/g and 10 μg/g were prepared.

2.2. Food/beverage samples

The sampling plan was designed in order to cover as much as possible the different food commodities listed in the Annex III of Regulation 1334/2008 (EU, 2008). Twelve food categories were selected and five samples for each group were collected and analysed (see Table S1 in Supplementary information). Samples were collected in Dutch and Belgian supermarkets and stored as specified on the label until further processing.

Fish-, meat-, bakery-, breakfast-, processed-, and mint confectionary products, chewing gums, and soups were homogenised using liquid nitrogen to obtain a fine powder and stored in the freezer. Ready-to-eat products were milled and stored at room temperature. Dairy products and sauces were stored in the fridge, while beverages were kept at room temperature.

Tea samples were analysed as ready-to-drink beverages. As the brewing time has been demonstrated to have a considerable influence on the extraction efficiency of thujone (Walch et al., 2011), all the tea samples were brewed according to the following protocol. A bag of 2 g was immersed into 150 mL of water at 80 °C and left for 5 min. The bag was retrieved and the sample was cooled to room temperature before extraction.

2.3. Sample preparation

For solid or semi-solid samples, 2.5 g was weighed into a Greiner tube and spiked with 25 μL of the IS working solution. Then, 5 mL of ethyl acetate were added and the mixture was shaken head-over-head for 30 min at room temperature. Subsequently, 2 g of magnesium sulphate and 0.5 g of sodium chloride were added. The mixture was shaken by hand to induce phase separation and centrifuged for 10 min at 3500 rpm. Half a millilitre of supernatant was filtered in a mini-uniprep PTFE filter vial (0.45 μm; Whatman, Buckinghamshire, UK) and analysed by GC–MS.

For beverages, an aliquot of 20 mL was spiked with 25 μL of the IS working solution. In case of alcoholic beverages, 20 mL of a 10-fold water-diluted sample was taken. Five milliliters of ethyl acetate were added and the mixture was shaken head-over-head for 30 min at room temperature. After the addition of 4 g of magnesium sulphate and 1 g of sodium chloride, the procedure continued as described for solid and semi-solid samples.

2.4. Instrumental method

A GC/MS system consisting of a gas chromatograph Bruker 450-GC (Bruker Daltonics B.V., Wormer, the Netherlands), equipped with a 1079 PTV programmable temperature vapourising injector and a CP-8400 autosampler, and coupled to a Bruker 300-MS mass spectrometer was used. The MS operated in single quadrupole mode.

The analytical GC column was a Restek Rtx®-CLPesticides 30 m × 0.25 mm internal diameter and 0.25 μm of film thickness (Interscience B.V., Breda, the Netherlands). The temperature program of the oven was as follows: 60 °C maintained for 1 min, then raised at 50 °C/min up to 80 °C, raised at 3 °C/min up to 125 °C and finally raised at 10 °C/min to 300 °C and maintained for 5 min. 1 μL of extract was injected in splitless mode. The injector temperature was set at 250 °C. The flow of helium was constant at 1 mL/min. The enantiomer separation of pulegone was carried out on a Restek Rt-bDEXsm 30 m × 0.25 mm; 0.25 μm of film thickness.

The MS was operated in electron impact (EI) mode. The temperature of the interface and the ion source were set at 280 and 250 °C, respectively. The acquisition of the data was conducted in both SCAN and SIM mode. The scan range covered 50–250 *m*/*z* with 0.2 s of scan time. Two ions (one quantifier and one qualifier) for every analyte were selected in SIM mode.

Data processing was performed with the software MS Workstation version 7.0 from Bruker.

2.5. Method validation

The method was validated according to the principles of the SANCO guideline for method validation and quality control of residues of pesticides SANCO/12571/2013 (SANCO, 2013).

In order to validate the method for the number of matrices listed in Regulation (EC) No. 1334/2008 (EU, 2008), four matrices, which represent the different types of foods (liquids, semi-solid, dry-solid and fatty-solid matrices) were selected. The performance parameters assessed were: limit of quantification (LOQ), selectivity, matrix effects, linearity, precision and recovery.

One blank material, previously assessed, was chosen for each matrix in the validation study. The blank materials were: distilled water for liquids, tomato sauce for semi-solids, grinded oat flakes for dry-solid matrices and mature cheese for fatty-solid matrices.

The method was validated in the range of 0.5–200 mg/kg for solid and semi-solid samples and of 0.0625–25 mg/kg for non-alcoholic beverages. For alcoholic beverages the range was increased ten-fold to 0.625–250 mg/kg. The concentration of the IS in the final extract was 5 μg/mL. The linearity requirements were fulfilled when the correlation coefficient was greater than 0.99 and the back-calculated concentration of the calibration standards did not exceed ±20% of the theoretical value.

The blank matrices were spiked at three different levels: LOQ level (0.5 mg/kg for solid/semi-solids or 0.0625 mg/kg for liquids), low-level (1 mg/kg or 0.125 mg/kg) and high-level (160 mg/kg for solids/semi-solids or 20 mg/kg for liquids).

The selectivity was assessed by verification of the absence of a significant signal (>30% of the LOQ) for the quantifier ion in blank sample extracts. Matrix effects were evaluated by comparing the response of each target analyte in the calibration standard prepared in solvent with the response obtained for the calibration standard prepared in matrix extract.

The precision, expressed as repeatability $\%RSD_r$, was assessed in sixfold at three different spiking levels. For each target analyte an in each matrix, RSD_r had to be less than or equal to 20% and only one outlier (Grubb's test) was allowed.

Due to the absence or certified reference materials, the trueness was assessed by the recovery of the spiked samples. The recovery was considered acceptable within the range of 70–120%.

LOQ was defined as the lowest level for which the requirements of repeatability and recovery were met.

2.6. Quality control during analysis of samples from the survey

Procedural blanks, sample blanks and quality control samples were run with each batch. Two criteria were used to ensure the correct identification of the target analytes: (a) the retention time of the analyte in the sample extract matched those of the analytes

in the standards within a deviation of ±0.2 min; (b) the similarity of the deconvoluted spectrum of the analyte in the sample compared to the spectrum of the analyte in the library should be at least 700.

3. Results and discussion

3.1. Optimisation of the sample treatment procedure

Methods to be applied for enforcement should comply with some requirements in order to be implemented for routine and control purposes. For example, sample preparation is recommended to be fast, straightforward, inexpensive, and versatile. Solvent extraction is hence a sample preparation technique that fulfils these prerequisites.

The solvent extraction procedure was optimised with regard to the type (acetonitrile, cyclohexane, ethyl acetate and methyl-*tert*-butyl ether) and volume (5–15 mL) of solvent, extraction time (5–60 min) and the addition of water.

Alcohols (e.g. methanol or ethanol) were excluded since they could react with aldehyde groups through aldol reaction (Lv et al., 2010). Toluene was rejected as extraction solvent due to its high molecular mass (MW = 92) and its high boiling point (111 °C). Chlorinated solvents and *n*-hexane were avoided due to their harmful and toxic properties. Therefore, and due to their polarity and their common application for liquid extraction of volatile compounds, the extraction efficiency of acetonitrile, cyclohexane, ethyl acetate and methyl-*tert*-butyl ether was evaluated.

For testing the extraction efficiency, a sample matrix with incurred target substances was used. For this purpose pesto, which is likely to contain both methyl eugenol and estragole was selected. The tests were performed in triplicate. Acetonitrile showed the lowest extraction yield (Fig. SS2 Supplementary information). Other disadvantages of acetonitrile versus either ethyl acetate or cyclohexane were its higher price and its greater toxicity. The differences in extraction yield when using methyl-*tert*-butyl ether, ethyl acetate or cyclohexane were not statistically significant. Ethyl acetate, with an octanol–water coefficient of 0.73 and water solubility of 8000 mg/L, was better able to penetrate into the high moisture samples than cyclohexane (octanol–water coefficient of 3.44 and water solubility of 55 mg/L) and was expected to be more favourable for extraction of non-polar analytes. Methyl-*tert*-butyl ether was more prone to losses due to evaporation and co-extracted other matrix constituents that interfered with the chromatographic measurements. Based on these finding, ethyl acetate was selected as extraction solvent.

The addition of water prior to the sample extraction is recommended to improve recoveries of hydrophobic pesticides/mycotoxins/plant toxins and other contaminants from low moisture containing commodities, such as cereals, spices or dried fruits. However, this treatment did not considerably enhance extraction yields of the target analytes (data not shown).

The amount of extraction solvent (5–15 mL) and the extraction time (5–60 min) were optimised to obtain maximum signal using central composite face (CCF). CCF is commonly used in the Engineer field, since it allows more information to be obtained from fewer experiments. A total of 11 experiments were carried out on either incurred samples (spices) or blank matrices spiked at 10 mg/kg with the target compounds. The results, which were corrected for the dilution effect, were similar for the various matrices tested and revealed that the volume of solvent was the only significant factor. The extraction time had no influence, which indicated that the equilibrium was reached rapidly (Fig. SS3 Supplementary information). Then, it was decided to use 5 mL of extraction solvent and an extraction time of 30 min.

Salts ($MgSO_4$/NaCl) were added to improve the recovery of the more polar analytes, such as *trans*-cinnamaldehyde and coumarin. $MgSO_4$ binds large amounts of water and significantly reduces the water phase that can be dissolved in ethyl acetate, thus promoting partitioning of the target analytes into the organic solvent layer. An aliquot of 4 g $MgSO_4$ was used for the 20 mL of liquid samples. This amount was reduced to 2 g when analysing 2.5 g of solid and semi-solid samples to prevent the formation of conglomerates. The addition of NaCl favours phase separation. The relative amount of $MgSO_4$/NaCl was 4:1, based on literature (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003).

3.2. Influence of alcohol in the extraction of alcoholic beverages

Ethanol may exert a highly significant impact on both the extraction efficiency and the chromatographic performance by distorting the peak shape of the analytes. As indicated in the specific ternary diagram (Griswold, Chu, & Winsauer, 1949) for ethyl acetate/water/ethanol, the composition in weight for the worst case scenario, absinthe with 80% of alcohol, would be 5 mL of EtOAC, 16 mL of ethanol and 4 mL of water (20:64:16) and would fall into the miscibility area, although the addition of salt might induce phase separation.

Therefore, the effect of the ethanol content of beverages on the extraction efficiency was evaluated. Samples containing 0%, 15% and 45% of ethanol were prepared in triplicate and spiked at 100 mg/kg with various of the regulated analytes and the IS. As shown in Fig. 1, while no significant differences between 0% and 15% ethanol were observed for almost all the target analytes, the extraction efficiency dropped considerably when ethanol increased from 15% to 45%. Furthermore, the presence of ethanol at 45% caused skewed peak shape in the GC analysis. The only compound for which the extraction efficiency was reduced by half in the presence of 15% ethanol, was coumarin. Coumarin, with an octanol–water coefficient of 1.39 and water solubility of 1900 mg/L, is soluble in ethanol, which enhances its partition to the aqueous phase.

Based on this results, it was decided to dilute alcoholic beverages 10-fold before extraction. The maximum amount of ethanol in the samples for the worst scenario would be 8%. The feasibility of this approach was confirmed with an additional experiment, in which samples mimicking 10-fold diluted alcoholic beverages were prepared and spiked with the target analytes at 0.5 mg/kg. The results (data not shown) confirmed that the concentration of ethanol did not have a statistically significant influence either on the recovery or on the chromatographic peak shape.

3.3. Validation of the method: performance characteristics

3.3.1. Linearity and matrix effect

The linearity in solvent and in matrix was assessed in the range corresponding to 0.5–200 mg/kg for solid and semi-solid samples, 0.0625–25 mg/kg for non-alcoholic beverages and 0.625–250 mg/kg for alcoholic beverages. The splitting of the calibration curves in at least two independent regressions was required to be compliant with the linearity criterion (see Section 2). One of the regressions was often suitable for high concentrations (10–200 mg/kg semisolids and solids and 2.5–25 mg/L for non-alcoholic beverages), while the other one usually matched low concentrations (0.5–10 mg/kg and 0.0625–2.5 mg/L respectively).

For the four representative food commodities, the matrix effect was assessed by comparing the response of the analyte in a solvent standard with the response of the analyte in a matrix-matched standard. In the liquid matrix, no significant matrix effect (<10%) was observed and, therefore, for validation and sample analysis of beverage samples, calibration was done using solvent standards. For the semi-solid and solid matrices, matrix effects were observed at the low concentrations and, therefore, for those samples, the

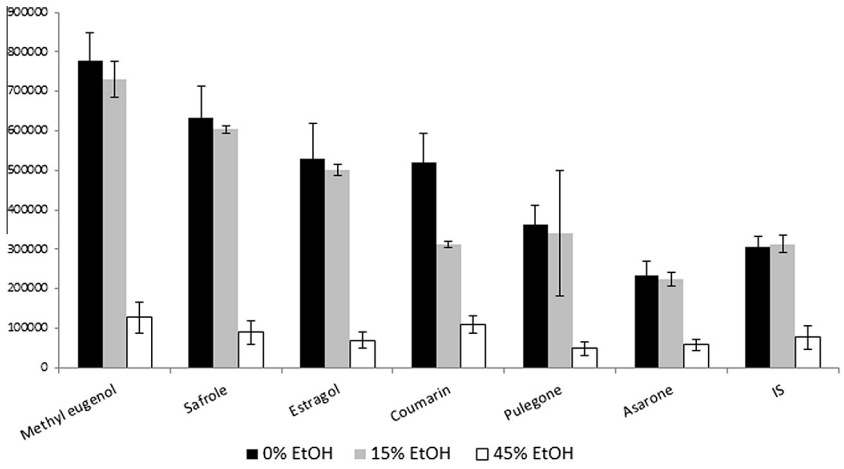

Fig. 1. Influence of the content of alcohol on extraction efficiency ($n = 3$). *y*-axis: response chromatographic area.

validation and the calibration were conducted using matrix-matched standards. Menthofuran showed matrix effect regardless of the type of matrix under study.

3.3.2. LOQs, recovery and repeatability

The results for repeatability and recovery are shown in Tables 1 and 2. LOQ-values, which were defined as the lowest level of spiking at which the requirement of repeatability was compliant, are shown in the Supplementary information. They differed slightly depending on the actual concentration of the analyte in the spiking standards, but were approximately 0.05 mg/kg for the non-alcoholic beverages and 0.5 mg/kg for the other food matrices.

In general, with the exception of menthofuran, the recovery fell with the criterion range of 70–120% and the repeatability was lower than 20%.

The behaviour of menthofuran in this method is not fully understood. Due to their similar chemical structure, menthofuran might undergo the same type of oxidation reaction as its structure-analogue curzerene (Zhao et al., 2011) when it is exposed to water and high temperatures. The natural occurrence of different menthofurolactones in *Mentha piperita* L. essential oil, as by-product of menthofuran oxidation, has been demonstrated by Frerot, Bagnoud, and Vuilleumier (2002). In the presented method, elevated temperatures (up to 40–45 °C) were reached when adding $MgSO_4$ to induce phase separation. $MgSO_4$ may also enhance the oxidation reaction.

3.3.3. Selectivity

The selectivity was evaluated for the quantifier ion of each analyte in the four representative food matrices. Despite the fact that solvent extraction and GC single quadrupole MS was used, which provide a lower degree of selectivity compared to headspace combined with GC–MS/MS (Bousova et al., 2011), the magnitude of the interference was never higher than 10% of the LOQ in all four matrices. An interference of 12% from the reagent was observed for carvacrol, which could bias the quantification at low concentrations. Matrix background was observed in case of cheese matrices, but it only affected the quantification ion of pulegone and thujone (m/z 81) at low concentrations.

3.4. Food survey

The broad range of food commodities for which maximum limits of flavouring substances are established made the design of a survey with a limited number of items challenging. The goal of this

Table 1
Recovery (%) and repeatability for the liquid and semi-solid matrices.

	Liquid matrices (water)						Semi-solid matrices (tomato sauce)					
	Spiking level 0.0625 mg/kg		Spiking level 0.125 mg/kg		Spiking level 20 mg/kg		Spiking level 0.5 mg/kg		Spiking level 1 mg/kg		Spiking level 200 mg/kg	
	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
trans-Anethole	53	(19)	53	(15)	85	(8)	102	(3)	105	(2)	113	(4)
Coumarin	111	(3)	111	(5)	84	(6)	97	(2)	102	(3)	112	(3)
Myristicin	108	(5)	96	(6)	85	(8)	105	(1)	104	(3)	107	(2)
Thujone	107	(10)	114	(16)	85	(7)	97	(4)	107	(2)	114	(4)
[a]β-Asarone	86	(11)	78	(7)	84	(7)	108	(1)	107	(3)	109	(3)
Methyl eugenol	110	(5)	100	(5)	84	(8)	107	(2)	106	(2)	110	(3)
Estragole	105	(18)	112	(20)	85	(7)	101	(3)	106	(2)	115	(4)
Menthofuran	n.d.		n.d.		20	(20)	60	(6)	70	(5)	61	(18)
Safrole	103	(7)	101	(10)	85	(8)	103	(3)	104	(2)	112	(4)
8-MOP	94	(2)	107	(4)	85	(6)	105	(8)	108	(5)	109	(8)
Elemicin	114	(5)	108	(10)	83	(10)	107	(1)	105	(3)	108	(2)
Pulegone	106	(9)	102	(13)	80	(18)	98	(3)	104	(2)	114	(3)
Thymol	102	(4)	99	(7)	87	(7)	101	(2)	104	(2)	116	(4)
Carvacrol	89	(7)	117	(5)	88	(8)	122	(4)	119	(3)	114	(3)
Cinnamaldehyde	102	(5)	100	(5)	85	(8)	100	(3)	104	(1)	114	(4)

[a] Concentrations for β-asarone: 0.016, 0.032 and 5.3 mg/L for liquid matrices and 0.1, 0.3 and 60 mg/kg for semi-solid matrices.

Table 2
Recovery (%) and repeatability for the solid matrices.

	Dry-solid matrices (oat flakes)						Fatty-solid matrices (cheese)					
	Spiking level 0.5 mg/kg		Spiking level 1 mg/kg		Spiking level 200 mg/kg		Spiking level 0.5 mg/kg		Spiking level 1 mg/kg		Spiking level 200 mg/kg	
	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
trans-Anethole	145	(9)	131	(11)	117	(6)	106	(11)	111	(5)	115	(7)
Coumarin	116	(7)	109	(3)	114	(5)	80	(3)	96	(5)	103	(8)
Myristicin	115	(7)	110	(1)	111	(5)	108	(3)	110	(6)	109	(4)
Thujone	120	(6)	140	(14)	126	(7)	110	(17)	109	(22)	125	(11)
[a]β-asarone	89	(19)	88	(9)	108	(5)	100	(4)	100	(10)	108	(7)
Methyl eugenol	121	(6)	125	(8)	114	(4)	98	(6)	109	(3)	111	(4)
Estragole	139	(11)	146	(13)	119	(7)	109	(17)	113	(11)	118	(8)
Menthofuran	n.d.		n.d.		n.d.		n.d		18	(24)	90	(14)
Safrole	130	(7)	126	(11)	116	(7)	108	(7)	112	(5)	113	(5)
8-MOP	111	(12)	104	(4)	111	(5)	87	(7)	101	(6)	111	(8)
Elemicin	127	(7)	110	(3)	116	(3)	95	(3)	108	(6)	109	(4)
Pulegone	132	(9)	137	(11)	119	(7)	107	(12)	112	(14)	116	(8)
Thymol	73	(18)	70	(4)	101	(7)	95	(5)	101	(4)	110	(7)
Carvacrol	66	(18)	64	(4)	96	(9)	87	(5)	99	(5)	107	(7)
Cinnamaldehyde	125	(4)	129	(9)	117	(6)	99	(10)	104	(4)	108	(6)

[a] Concentrations for β-asarone: 0.1, 0.3 and 60 mg/kg.

Table 3
Concentration levels (mg/kg) for the analytes of interest in the different food commodities.

Food commodities	Compound	# detected	Range (mg/kg)	Mean (mg/kg)	Median (mg/kg)	[a]Maximum limit (mg/kg)
Dairy products (n = 5)	Coumarin	1/5	(<[b]LOQ – 2.2)	2.2	2.2	[c]–
	Myristicin	3/5	(<LOQ – 19)	6.8	1.0	–
	Thujone	1/5	(<LOQ – 0.7)	0.7	0.7	–
	Methyl eugenol	1/5	(<LOQ – 1.4)	1.4	1.4	20
	Safrole	1/5	(<LOQ – 2.4)	2.4	2.4	–
	Elemicin	1/5	(<LOQ – 1.3)	1.3	1.3	–
	trans-Cinnamaldehyde	1/5	(<LOQ – 5.0)	5.0	5.0	–
Processed fruits, vegetables, nuts and seeds (n = 5)	Coumarin	1/5	(<LOQ – 6.1)	6.1	6.1	–
	Myristicin	3/5	(<LOQ – 1.5)	1.1	1.2	–
	Methyl eugenol	1/5	(<LOQ – 1.2)	1.2	1.2	–
	Estragole	2/5	(<LOQ – 5.2)	3.9	3.9	50
	Thymol	1/5	(<LOQ – 3.5)	3.5	3.5	–
	Carvacrol	1/5	(<LOQ – 41)	41	41	–
	trans-Cinnamaldehyde	1/5	(<LOQ – 15)	15	15	–
Fish products (n = 5)	Myristicin	1/3	(<LOQ – 1.3)	1.3	1.3	–
	Estragole	2/5	(<LOQ – 1.0)	0.9	0.9	50
	Thymol	2/5	(<LOQ – 5.8)	4.4	4.4	–
	Carvacrol	2/5	(<LOQ – 4.9)	2.6	2.6	–
Meat products (n = 5)	Myristicin	2/5	(<LOQ – 2.5)	1.9	1.9	–
	Methyl eugenol	1/5	(<LOQ – 0.6)	0.6	0.6	15
	Elemicin	1/5	(<LOQ – 1.2)	1.2	1.2	–
Soups and sauces (n = 6)	Myristicin	2/5	(<LOQ – 11.3)	6.4	6.4	–
	Estragole	1/5	(<LOQ – 2.1)	2.1	2.1	–
	Thymol	1/5	(<LOQ – 2.0	2.0	2.0	–
	Carvacrol	1/5	(<LOQ – 9.3)	9.3	9.3	–

Table 3 (*continued*)

Food commodities	Compound	# detected	Range (mg/kg)	Mean (mg/kg)	Median (mg/kg)	[a]Maximum limit (mg/kg)
Ready-to-eat savouries (n = 5)	Myristicin	3/5	(<LOQ – 2.5)	1.2	0.7	–
	Estragole	2/5	(<LOQ – 2.4)	2.3	2.3	–
	Thymol	4/5	(<LOQ – 6.8)	2.9	1.7	–
	Carvacrol	4/5	(<LOQ – 70)	20	4.0	–
Confectionary (n = 5) mint/peppermint containing micro breath freshening	Pulegone	3/5	(<LOQ – 6.2)	3.7	2.6	250 2000
Alcoholic beverages (n = 6) from *Artemisa* species (n = 2)	*trans*-Anethole	5/6	(<LOQ – 5090)	1642	738	–
	Coumarin	3/6	(<LOQ – 63)	23	4	–
	Thujone	2/6[d]	(<LOQ – 30)	18	18	10/35 (*Artemisa*)
	8-MOP	1/6	(<LOQ – 2.1)	2.1	2.1	–
	Thymol	1/6	(<LOQ – 4.1)	4.1	4.1	–
	trans-Cinnamaldehyde	2/6	(<LOQ – 44)	23	23	–
Non-alcoholic beverages (n = 5)	*trans*-Anethole	3/5	(<LOQ - 3.3)	1.3	0.4	–
	Coumarin	1/5	(<LOQ – 7.1)	7.1	7.1	–
	Thujone	1/5	(<LOQ – 2.7)	2.7	2.7	–
	Estragole	1/5	(<LOQ – 0.15)	0.15	0.15	10
	trans-Cinnamaldehyde	2/5	(<LOQ – 28)	15	15	–
Breakfast cereals containing muesli (n = 5)	Coumarin	2/5	(<LOQ – 11)	6.9	6.9	20
	trans-Cinnamaldehyde	2/5	(<LOQ – 53)	31	31	–
Chewing gum	*trans*-Anethole	3/5	(<LOQ – 1073)	364	19	–
	Pulegone	4/5	(<LOQ – 140)	75	73	350
	Carvacrol	1/5	(<LOQ – 0.7)	0.7	0.7	–
	trans-Cinnamaldehyde	1/5	(<LOQ – 31)	31	31	–
Bakery products (n = 5) without cinnamon (n = 1) with cinnamon (n = 4)	*trans*-Anethole	1/5	(<LOQ – 3.7)	3.7	3.7	–
	Coumarin	[e]4/5	(<LOQ – 15)	8.5	9.1	50
	Myristicin	2/5	(<LOQ – 4.8)	3.2	3.2	–
	Methyl eugenol	3/5	(<LOQ – 3.3)	1.8	1.1	–
	trans-Cinnamaldehyde	[e]4/5	(<LOQ – 19)	9.9	10	–

[a] Regulation 1334/2008.
[b] See Supplemental information for LOQ.
[c] Maximum limits not available.
[d] The two positive samples for thujone were the two absinthe samples.
[e] All positive samples for coumarin also contained *trans*-cinnamaldehyde.

first survey was to cover all the food commodities that are mentioned in Regulation (EC) 1334/2008.

A survey of a total of 61 different food commodities assigned to 12 groups was conducted. Five items per group were sampled and analysed with the validated method. The detection frequency and concentrations range for all target analytes are provided in Table 3. The concentrations for the flavouring substances for each individual sample from the survey can be found in the Supplementary information.

The EU regulated flavouring substances were found in thirty-one food samples, all within their respective maximum limits. The non-regulated flavourings were detected in thirty-four food samples at concentrations comparable to those reported in other studies (Avila et al., 2009; Bousova et al., 2011; Lachenmeier et al., 2008; Lungarini et al., 2008; Raffo et al., 2011; Siano et al., 2005, 2003; Sproll et al., 2008; Walch et al., 2011).

β-Asarone, the active ingredient found in the plants *A. calamus* (calamus) and *Acorus europaeum* (hazelwort), was not detected in any of the samples. In published studies, β-asarone was detected at levels up to 0.35 mg/kg in vermouths and up to 4.96 mg/kg in a selection of alcoholic beverages (SCF, 2002b) but no recent data on β-asarone occurrence in alcoholic beverages are available.

Pulegone (Fig. 2A) was detected in three samples of mint/peppermint containing confectionary at levels around 5 mg/kg and in the mint-flavoured chewing gums. A large variability in the concentrations detected for pulegone was found in mint-flavoured chewing gum samples, ranging from 13 to 140 mg/kg. The higher levels could be due to the extended manufacturing practice of

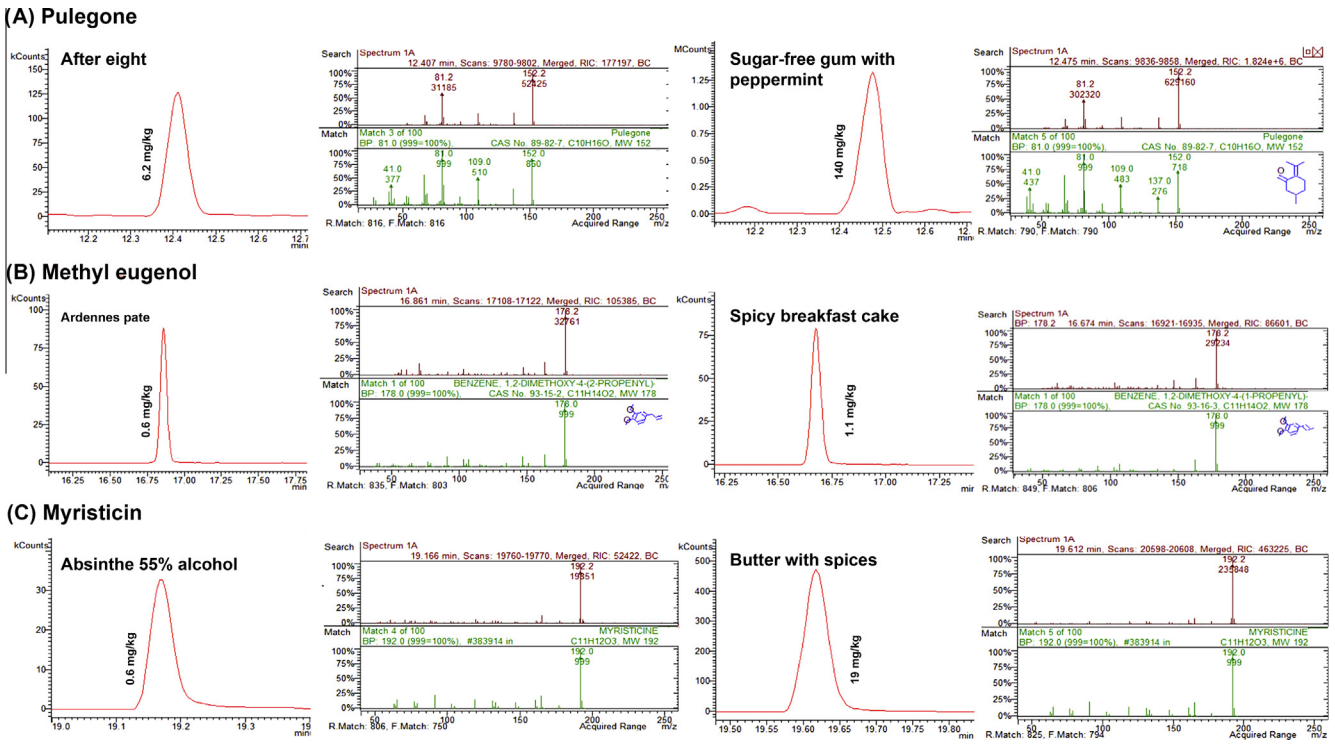

Fig. 2. Identification of pulegone, methyl eugenol and myristicin in various samples.

adding flavour to strengthen the 'peppermint' aroma. The values found in products from the Dutch retail market were in agreement with other European studies (Siano et al., 2005). The enantiomeric analysis of pulegone deserves particular consideration since it may serve to detect the natural source of the flavouring and/or possible adulterations or counterfeits. (R)-(+)-pulegone, together with (R)-(+)-menthofuran, is the main constituent of peppermint and pennyroyal oil, while (S)-(−)-pulegone is present in Buchu leaf oil, produced from steam distillation of *Agathosma betulina* leaves that is often used to provide "cassis" type aroma (Siano et al., 2005). The analysis of the samples on a chiral column revealed that only the (R)-(+) enantiomer was present, thus indicating that only mint/peppermint essential oils were used as flavourings.

Thujone occurred in ready-to-drink sage tea at 2.7 mg/kg (*Salvia officinalis*) and in the two absinthe beverages at 5 and 30 mg/kg (made of *Artemisia* ssp.). Both diasteroisomers, α and β-, were detected and their ratios differed depending on the plant of origin. α-Thujone (85%) was the predominant isomer in the sage tea sample (Fig. 3A), while β-thujone (60–70%) was more abundant in absinthes beverages (Fig. 3B), which was in agreement with the reported composition for the essential oils of *S. officinalis* (Perry et al., 1999) and *Artemisia* ssp. (Lachenmeier, Walch, Padosch, & Kroner, 2006). The European food law recently deregulated the use of thujone-containing plants in food. The previous limits of 25 mg/kg for foodstuffs containing preparations based on sage, and of 5 mg/kg for any type of food, have been eliminated and only *Artemisia*-containing beverages are currently of concern (EU, 2008). This means that *S. officinalis* and other thujone-containing flavouring plants (besides *Artemisia*) can now be used in foods without restrictions. However, the opposite is true for herbal medicines. The European Medicines Agency (EMA) has recently proposed an acceptable daily intake (ADI) of 6.0 mg/person/day of thujone (EMA, 2011). These regulatory differences lead to the strange anomalies: there are no limitations in consuming sage tea, while intake from herbal medicine is restricted (although, in this particular sample, 15 cups/day would have to be consumed to reach the ADI).

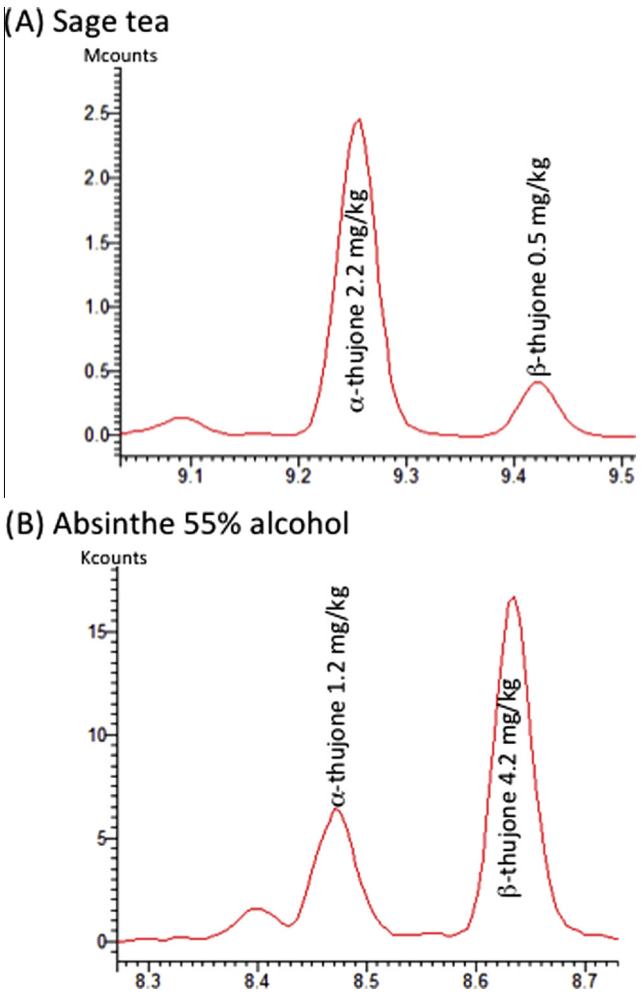

Fig. 3. Diasteroisomeric distribution of α and β-thujone in sage tea and absinthe.

The two absinthe beverages contained *trans*-anethole in amounts ranging 1460–5090 mg/kg, which lead to the conclusion that fennel and/or anise may have been used in the manufacturing of absinthe as taste improvers (Lachenmeier et al., 2006). The recipe for absinthe preparation differs in every country; e.g. in Czech Republic peppermint is usually added instead of anise or fennel. *trans*-Anethole was also detected in other alcoholic liquors (1.2–5.3 mg/kg), fennel tea (3.3 mg/kg), one bakery product (3.7 mg/kg) and three chewing gums (0.5–1073 mg/kg). Although the Joint FAO/WHO Expert Committee on Food Additives (JECFA) derived an ADI of 0–2.0 mg/kg bw/day for *trans*-anethole (about 0–120 mg/person/day), they concluded that there is no safety concern at current levels of intake when used as flavouring. Furthermore, the intake of four cups per day of fennel tea would imply a consumption of 1.95 mg of *trans*-anethole and also 88 μg of estragole, which is well below the estimated daily exposure of 28–98 mg for *trans*-anethole and 1.9–5.8 mg for estragole by EMA (EFSA, 2009).

Coumarin occurred within the range 0.6–63 mg/kg in black tea with cinnamon, three alcoholic beverages, in quark cheese with cinnamon and apple, in apple sauce with cinnamon, in four desserts and in three breakfast cereals. Regulatory limits are established in only the last two food commodities (EU, 2008). Based on a tolerable daily intake (TDI) of 0.1 mg/kg bw/day, the concentrations of coumarin for even the non-regulated products would be of no concern for adults: the TDI would be only exceeded if large quantities of these products were consumed on daily basis. However, the scenario is different for more sensitive populations like children (20 kg). The intake of either 130 g of breakfast cake or a whole packet of crisp would imply an exposure higher than recommended. Cinnamaldehyde was present in all products that contained coumarin at higher levels than previous studies (Lungarini et al., 2008). Cinnamaldehyde is the most abundant compound of cinnamon and its simultaneous occurrence with coumarin supports the BfR opinion, which concluded that consumers of large quantities of cinnamon have a high exposure to coumarin (BfR., 2006). BfR also proposed that the restriction in the dietary intake of coumarin should be maintained. JECFA has promoted several toxicological assessments of cinnamaldehyde as a food ingredient in the last decades. At its 35th meeting, the ADI of 0.7 mg/kg bw/day was not extended and the flavouring was stated as of no safety concern. However, cinnamaldehyde has been shown to be a genotoxic agent in humans (Lungarini et al., 2008).

Estragole, methyl eugenol and safrole have been demonstrated to be genotoxic and carcinogenic; hence safe exposure limits cannot be established (CoE, 2005). In addition to fennel tea, estragole was detected at the range of 0.7–5.2 mg/kg in two fish samples, two Italian herbs-containing ready-to-eat savouries, two herbs-containing processed vegetables and in one pesto. Only one sample (butter with spices) contained detectable levels of safrole (2.4 mg/kg). Methyl eugenol (Fig. 2B) was identified in the range of 0.6–3.3 mg/kg in three samples of fine bakery products, a meat sample, one dairy product and one herb-containing processed vegetable. An Italian homemade pesto was also analysed as a comparison with the industrial products. It contained 185 mg/kg of methyl eugenol, which exceeds the maximum permitted level for soups and sauces by three times. The regulatory limit is not applicable in this particular scenario since it specifies that the maximum levels do not apply when estragole, methyl eugenol and safrole are added as either fresh, dried or frozen herbs and spices (EU, 2008). Pesto is a typical Italian basil-based pasta sauce that originally comes from Liguria, a North Italian region. Only raw pesto preserves the composition of its ingredients. In contrast, food industries manufacture pesto by applying thermal treatment to the raw ingredients, while mild preservation techniques like moderate atmosphere packaging or refrigeration are only used for small and local producers (Zunin, Salvadeo, Boggia, & Lanteri, 2009). The other three samples of Genovese pesto analysed were heat-processed to preserve their quality for longer, which may have caused losses in the volatile fraction that contained estragole and methyl eugenol.

The food commodities containing Italian herbs or spices were also positive for the presence of thymol and carvacrol in a range 1.2–6.8 mg/kg for thymol and 0.5–70 mg/kg for carvacrol. Their presence was common in almost all ready-to-eat savouries. Carvacrol and thymol are the most abundant constituents of oregano and thyme (Lopez et al., 2007), which are – together with basil – the most commonly known Italian herbs.

Myristicin and elemicin are alkenylbenzenes that are not regulated in EU (EU, 2008), although they have been assessed as genotoxic in the available literature (van den Berg et al., 2011). Myristicin (Fig. 2C) was detected in sixteen samples including meat, fish, sauces, processed vegetables, ready-to-eat savouries and desserts, ranging from 0.6 to 19 mg/kg, while elemicin was detected only in butter with spices at 1.3 mg/kg and in pate at 1.2 mg/kg. No TDIs have been determined for either of them. Their occurrence is restricted to the intake of safrole in nutmeg-containing foods (CoE, 2005). EFSA recommends applying the Margin of Exposure (MOE) approach for the risk assessment of carcinogenic and genotoxic compounds occurring in foods and states that a MOE of 10,000 or higher would be considered as a low priority for risk assessment (EFSA, 2005a). Recent estimates of dietary exposure of the European population to myristicin, via the consumption of spices of nutmeg and its oil, have established *per capita* mean values for lower/upper limits of 162 μg and 3684 μg/day (Raffo, D'Aloise, Magri, & Leclercq, 2013). However, as demonstrated here, myristicin can be detected in products besides those containing nutmeg and its presence is not directly linked to the occurrence of safrole.

4. Conclusions

A fast, straightforward, inexpensive, and versatile method was developed and successfully validated to determine the occurrence of seven alkenylbenzenes and seven other flavouring compounds in a broad range of food commodities. The levels at which this method was validated make it suitable to enforce the Regulation (EC) 1334/2008.

The method was applied in a survey of foodstuff coming from the Dutch or Belgian retail market. This survey comprised 61 food commodities in 12 categories, which were intended to be in agreement with the food items listed in the Regulation (EC) 1334/2008. All products were compliant with the maximum permitted limits in EU. The concentrations of coumarin, which commonly occurred in some breakfast and bakery products and other cinnamon-containing food commodities, did not constitute a health risk for adults based on the ADI appointed by JECFA. However, the levels detected in this study may exceed the TDI established by EFSA for children.

Myristicin and *trans*-anethole, for which no legal limits are set, were the most common alkenylbenzenes detected in the Dutch food products. Myristicin was frequently present in nutmeg- and herbs-containing products, while *trans*-anethole usually occurred in fennel-containing products. Taking into account this survey, monitoring of myristicin and *trans*-anethole, together with elemicin, is recommended to gain insight into consumer exposure to these alkenylbenzenes with toxic and carcinogenic/mutagenic properties.

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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.foodchem.2014.11.011.

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