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Journal of Chromatography B, 678 (1996) 205–210

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Gas chromatographic–mass spectrometric method to characterise the transfer of dietary odorous compounds into plasma and milk

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Received 8 May 1995; revised 10 November 1995; accepted 17 November 1995

Abstract

The flavours contained in a mammalian mother's milk can exert a marked influence on her offspring's proximate suckling behaviour and later preferences. The aim of this study was to establish a reliable analytical procedure to characterise the mammary transfer of selected volatile constituents of maternal food from non-pregnant and recently parturient ewes. Six known volatile compounds, most representative of cumin aroma (α-pinene, γ-terpinene, cuminaldehyde, *p*-cymene, limonene and cineole), were traced in the blood and milk of ewes fed with cumin seeds, using liquid–liquid extraction combined with gas chromatography–specific ion monitoring mass spectrometry. Among the six cumin odour markers, only one, *p*-cymene, was transferred in quantifiable amounts into the venous plasma. The other cumin markers could only be detected as traces corresponding to amounts lower that the limit of quantification. In milk, four of the cumin markers could be detected, and two of these were quantified

Keywords: Cumin odour markers; α-Pinene; γ-Terpinene; *p*-Cymene; Limonene; Cineole; Cuminaldehyde

1. Introduction

The flavours contained in a mammalian mother's milk can exert a marked influence on her offspring's proximate suckling behaviour and later preferences. Suckling promotes odour/taste associations that are particularly strong and lasting over time, as they can be retained till weaning and then bias the ingestive preferences of the adult organism [1]. As yet, the transmission of chemosensory information through maternal milk, and the learning of this information by neonates has been examined mainly in rodents (rat [2–5]; mouse [6]; lagomorphs [7]). Whether such very precocious mother-to-infant transmission of chemical cues is also functional in other mammalian groups is poorly understood

This study presents a first step in the documentation of the transfer of odour-active compounds contained in the mother's diet into her bloodstream and milk, in sheep. Its aim was to establish a reliable analytical procedure for the characterisation of the the rate and kinetics of mammary transfer of selected

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0378-4347/96/$15.00
SSDI 0378-4347(95)00527-7

volatile constituents of aromatic nutrients in the lactating ewe. The procedure selected for tracing dietary aromas in plasma and milk was liquid–liquid extraction combined with GC–specific ion monitoring MS.

2. Experimental

2.1. Animals, chemicals, odorous molecules

Three non-pregnant and two recently parturient (postpartum day 7), lactating ewes (Ile-de-France breed) were used for this study. These ewes were housed in individual pens, so that their effective ingestion of the flavoured dietary rations could be controlled.

Cumin (*Cuminum cyminum L.*) was used as the source of aromas. The rationale for choosing this spice was that: (i) it is readily accepted by ewes; (ii) it is a seasoning abundantly used in numerous cuisines around the world, and in certain cultures, it is even prescribed to favour lactation in new mothers [8]; and (iii) some of the odorous compounds of cumin are known to be easily transferred to amniotic fluid and milk in pregnant women [9]. The known volatile compounds most representative of cumin seed aroma were selected to be traced in blood and milk. The selected constituents of the cumin aroma were: β-pinene, γ-terpinene, cuminaldehyde, *p*-cymene, limonene and cineole. The proportions of these compounds in fresh seeds are 20.1, 18.5, 16.4, 11.1, 11.1 and 11.1%, respectively [10–12]. For analytical purposes, β-pinene, γ-terpinene, cuminaldehyde, *p*-cymene, limonene and cineole were purchased as pure compounds from Aldrich Chemicals (Saint Quentin Fallavier, France).

2.2. Ingestion of dietary aromas and sampling procedure

Cumin seeds (250 g, African ecotype, purchased at La Bovida, Paris) were administered in a single dose, mixed with the daily grain ration. The cumin dose was relatively high in order to optimise the presence of volatile aromas after the ruminal processing. All sheep entirely consumed their cumin–grain mix, but at different rates.

Two types of biological samples were collected from the ewes: blood punctured from the jugular vein and milk obtained by milking. The samples of plasma were collected for 49 h, using B-D Vacutainer vials (Na-heparin-lined). Blood samples were centrifuged shortly after puncture (3000 *g* at 4°C for 30 min) and plasma samples were frozen at −20°C. Milk was collected according to the same timing protocol and was frozen immediately.

2.3. Extraction

The biological matrices were submitted twice to a liquid–liquid extraction using petroleum ether (40–60°C fraction, Merck, Darmstadt, Germany). Five milliliters of the matrix (blank spiked with the target compounds or collected from the ewes) were extracted with 15 ml (for plasma) or 10 ml (for milk) of petroleum ether in a 45-ml PTFE-capped tube by careful manual agitation, in order to avoid the formation of an emulsion. If the sample was emulsified, the emulsion was broken by sonication for 3 min in an ice bath. After centrifugation (1700 *g* for 10 min at 5°C), the upper layer was separated and stored in a 20-ml tube. The residue was submitted to a further extraction with an additional 6-ml volume of petroleum ether, under the same conditions. The shaking time was reduced to 2 min. The two extracts were combined and concentrated, by evaporation under a stream of nitrogen, in an ice bath and adjusted exactly to a 1-ml volume with solvent after equilibration of the sample at room temperature. The sample was transferred to an auto-sampler vial. A 3-μl volume of this sample was injected automatically according to the chromatographic conditions described in Section 2.5.

2.4. Calibration

Calibration series were prepared either by mixing the six target compounds in petroleum ether (direct series) or by extracting 5 ml of plasma or milk with the same solvent (extracted series); both matrices were added to the six target compounds at the following concentrations: 0.1, 0.25, 1, 5, 10 ng/μl. The calibration series were prepared from 5-ml aliquots of plasma or milk from control animals

(never fed with cumin). The six cumin aroma markers were introduced into these aliquots by adding a constant volume (100 μl) of methanolic solutions in order to obtain final concentrations of 20, 50, 200, 1000 and 2000 μg/l for each target compound.

For each concentration series, the extraction of the non-flavoured (control) matrix provided the baseline. Comparison of the direct and extracted series allowed us to verify that the target compounds were not lost during the extraction process of the sample.

The linearity of the analytical responses was estimated by a linear regression on the area of the characteristic signal of each target compound (mass chromatogram of the characteristic ion) and the concentration of that compound.

2.5. GC–MS procedure

The cumin odour markers were detected and measured by mass spectrometry, after gas chromatographic separation on an apolar capillary column. The mass spectra obtained under electron impact from the mixture of the pure compounds allowed us to select three characteristic ions for each of them. These ions were monitored by specific ion monitoring (SIM), in order to measure the target compounds at very low concentrations. The chromatographic conditions were optimised to obtain a satisfactory resolution for *p*-cymene, limonene and cineole, and for their separation from the compounds extracted from both analysed matrices.

A Hewlett-Packard 5890 gas chromatograph, equipped with a splitless injector and a HP 5 capillary column (25 m × 0.2 mm I.D.) coated with a 5% phenylmethyl silicone phase (0.33 μm), was used. The chromatograph was coupled with a mass spectrometer (MSD HP 5970) working under electron impact. The samples were injected with an automatic auto-sampler (ALS 7673). The whole analytical procedure was controlled with the program MS 3.2 of a Pascal Chemstation (HP 59970).

The target compounds were diluted with petroleum ether at a concentration of 20 ng/μl and were injected first individually (1 μl) and then as a mixture, in order to optimise the chromatographic separation of *p*-cymene, limonene and cineole. Thus, the following elution conditions were used: (i) the temperature of the injection port was set at 260°C - splitless mode (valve time: 54 s); (ii) the oven temperature was programmed up to 280°C to elute the higher molecular mass compounds extracted from the matrix [35°C for 1 min; increased by 15°C/min up to 75°C (then held constant for 1 min); 3°C/min up to 90°C; 20°C/min up to 180°C; 25°/min up to 280°C (then held constant for 5 min); (iii) helium was used as the carrier gas, at a velocity of 35 cm/s.

3. Results and discussion

3.1. Characterisation of the cumin aroma markers

Fig. 1 shows a chromatogram of a mixture of the six cumin aroma markers in petroleum ether. For each compound, the recording of mass spectra that were in agreement with previous reports [13,14], allowed us to select a characteristic ion that could be used for quantification and two additional ions that could be used for certification of this compound. The criteria for certification were based on the overlap of the retention time of the three ions and on the agreement of their relative intensities with those reported for the mass spectrum of the reference compound. In Table 1, the ions monitored for the analysis and the expected ratios of the selected additional ions relative to the main ion used for quantification, are presented.

Limonene, *p*-cymene, and β-pinene cannot be quantified by using their low intensity molecular ions, so ions at *m/z* 93, 119 and 93, which provide a more intense signal, must be used. On the contrary, cineole and γ-terpinene can be quantified using ions at *m/z* 154 and 136, respectively, because their specificity is better despite a very intense ion at *m/z* 93. Finally, cuminaldehyde yields two ions (*m/z* 148 and 105), which are both specific and intense.

Using these ions, the mass fragmentometry method was set up following the procedure presented in Table 2. Fig. 2 shows the mass fragmentogram resulting from the plasma samples spiked with a mixture containing 50 ppb of the six target compounds. The specificity of ion detection increases the

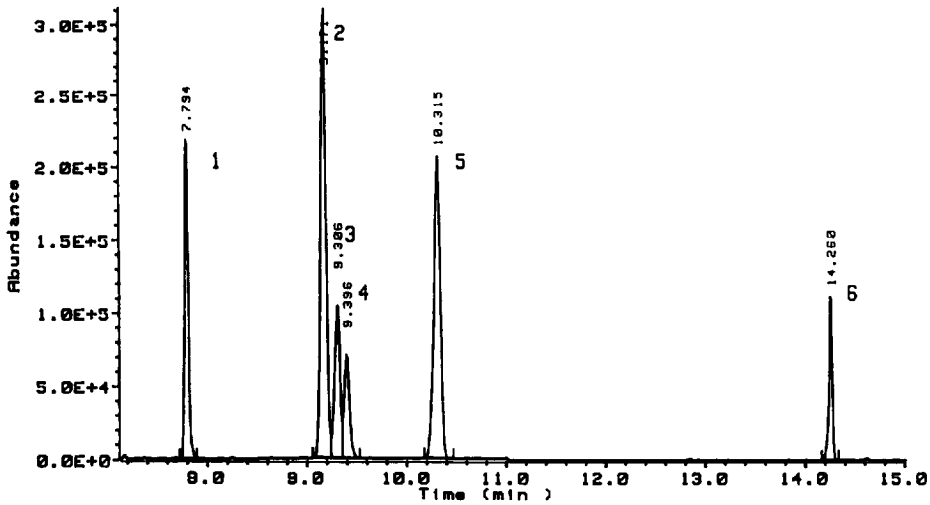

Fig. 1. Chromatogram of the six cumin aroma markers in solution at 20 ng/μl in petroleum ether. Peak numbers 1 to 6 are for: 1 = β-pinene; 2 = *p*-cymene; 3 = limonene; 4 = cineole; 5 = γ-terpinene and 6 = cuminaldehyde. Retention times are given at the top of the peaks.

Table 1
m/z Values of the ions monitored for quantification and additional ions with their relative abundance to the additional ion

Compound	Quantification ion *m/z* 1	Additional ion No. 1		Additional ion No. 2	
		m/z 2	%	*m/z* 3	%
β-Pinene	93	121	12	136	10
p-Cymene	119	134	25	91	35
Limonene	93	121	32	136	32
Cineole	154	139	100	93	140
γ-Terpinene	136	121	95	93	400
Cuminaldehyde	148	105	110		

resolution of compounds that are difficult to separate, such as *p*-cymene and limonene (ions at *m/z* 93 and 119), or cineole (ion at *m/z* 154).

3.2. Linearity

The results obtained, from both plasma and milk, for the calibration curves within the range 20–2000 ppb, showed that the responses are linear for all the series (r^2>0.999) and for each compound.

Table 2
Retention time windows and ions monitored for the quantification of the cumin markers

Time (min)	Ion group	*m/z* Values							Compounds
7.00	Group 1	93	121	136					β-Pinene
	Dwell time (ms)	50	50	50					
8.50	Group 2	93	119	121	134	136	139	154	*p*-Cymene
	Dwell time (ms)	50	50	50	50	50	50	50	Limonene
									Cineole
									γ-Terpinene
11.0	Group 3	105	148						Cuminaldehyde
	Dwell time (ms)	50	50						

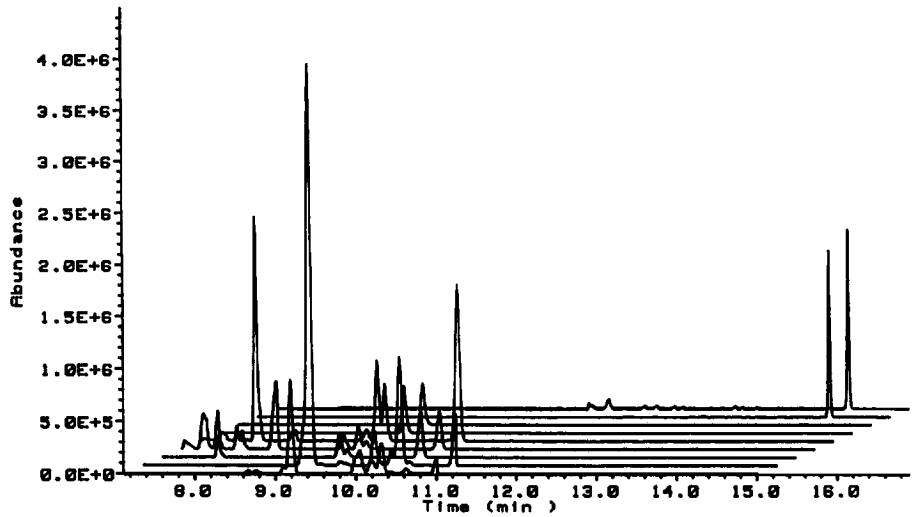

Fig. 2. Mass fragmentogram of a plasma sample spiked with 50 ppb of each aroma marker. Traces from front to back are for *m/z* 119, 91, 136, 121, 93, 154, 139, 148 and 105, respectively. The shift in the *x* direction is 5%.

3.3. Limit of quantification

The limit of quantification was fixed at 20 ppb, corresponding to the lower point of the calibrating range for all compounds, with the exception of cuminaldehyde whose limit of quantification was 50 ppb.

Due to the high lipid content of milk and, to a lesser extent, of plasma, as well as the use of a non-polar solvent extraction system, the analytical samples obtained prior to injection were extremely rich in potentially interfering apolar materials or in high molecular mass compounds. Moreover, the low level of detection did not allow for the use of the split injection mode. In the same way, the final sample volume could not be reduced down to less than 1 ml, without dramatically increasing the amount of interfering compounds introduced into the column. Thus, the injected amount of each compound (except for cuminaldehyde) at the lower point of the calibrating range was 300 pg, corresponding to a 5-ml matrix sample, a 1-ml analytical sample volume, and a splitless injection volume of 3 μl. To avoid the accumulation in the column of high molecular mass material, and to prevent peak shape degradation by compound adsorption, the oven temperature was raised to 280°C and then held for 5 min at the end of each run.

Nevertheless, the use of multiple detection on three representative ions of each compound, permitted the detection, with certainty, of the presence of some target molecules at levels significantly under the limit of quantification.

3.4. Transfer of cumin odorous markers into plasma and milk

Among the six cumin odour markers retained for analysis, only one (*p*-cymene) was transferred into the plasma in quantifiable amounts. The plasma concentration of *p*-cymene reached the detection limit 2 h after ingestion in all the ewes and mostly peaked after 17 h (Table 3). Individual differences were apparent in the evolution of *p*-cymene plasma concentrations, lactating ewes having lower levels than non-lactating ones. The other cumin markers could only be detected as traces corresponding to amounts lower than the limit of quantification.

The different target compounds could only be detected from one series of milk samples. From these samples, four of the six cumin markers could be detected, and two of these were sufficiently concen-

Table 3
p-Cymene plasma concentrations after cumin intake

Time (h)	Ewe number					
	82702	90723	42115	91467	61198	Mean
0	0	0	0	0	0	0
2	61.4	60.6	65.3	61.5	61.1	61.8
9	105.7	62.7	91.9	137.9	125.0	104.6
17	202.0	103.2	229.7	269.6	166.0	194.1
25	133.4	70.5	207.4	153.4	179.8	148.9
33	80.5	57.9	142.2	88.6	117.0	97.2
41	67.4	55.8	115.0	nd	86.9	81.3
49	62.4	79.8	37.1	nd	69.6	69.7
Maximum concentration	202.0	103.2	229.7	296.9	179.8	194.1

nd = not determined.

trated to allow quantification. In Table 4, the evolution of the concentration of these four compounds with time, is shown. It can be observed that β-pinene and *p*-cymene were transferred into milk in larger amounts than the quantification threshold of the method. The concentration of both volatile molecules peaked 9 h after ingestion and decreased thereafter to reach very low levels after 41 h. The transfer of γ-terpinene and cuminaldehyde was considerably lower and slower than that observed for some of the other compounds. Limonene and cineole could not be detected with the present extraction and detection techniques.

4. Conclusion

The analytical method developed for the quantification of the odorous markers of cumin, from plasma and milk, by GC–MS allows the quantification of these compounds down to 20 ppb from each kind of matrix. It is linear in the range 20–2000 ppb, starting from a 5-ml matrix sample and with 3 μl of injected extract. Liquid–liquid extraction has to be carried out very carefully in order to avoid the formation of emulsion, especially with milk samples.

Using this method, some of the volatile constituents of cumin selected as transfer markers were found in quantifiable amounts in both plasma and milk after a single intake of 250 g of cumin seeds. Under these conditions, limonene and cineole levels were always under the limit of quantification. In plasma, only *p*-cymene reached concentrations that were significantly above the quantification threshold. The SIM technique offers high specificity by judicious choice of representative ions, and the use of automatic injection with splitless mode, renders the analytical method fully automatic.

Table 4
Concentration of the various cumin markers in ewe's milk No. 90723

Compound	Time (h)							
	0	2	9	17	25	33	41	49
β-Pinene	<lq[a]	<lq	150	68	nd[b]	69	<lq	<lq
p-Cymene	<lq	<lq	141	46	<lq	<lq	<lq	<lq
Limonene	<lq	<lq	<lq	<lq	<lq	<lq	<lq	<lq
Cineole	<lq	<lq	<lq	<lq	<lq	<lq	<lq	<lq
γ-Terpinene	<lq	<lq	13	2	<lq		19	22
Cuminaldehyde	<lq	<lq	10	10	<lq	10	11	10

[a] <lq = below the limit of quantification.
[b] nd = not determined.

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