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Analytical Methods

Development of an HS-SPME-GC method to determine the methyl anthranilate in *Citrus* honeys

Davide Bertelli [a,*], Giulia Papotti [a], Massimo Lolli [a], Anna Gloria Sabatini [b], Maria Plessi [a]

[a] *Dipartimento di Scienze Farmaceutiche, Università di Modena e Reggio Emilia, via Campi 183, 41100 Modena, Italy*
[b] *Consiglio Nazionale per la Ricerca e Sperimentazione in Agricoltura – Istituto Nazionale di Apicoltura, via di Saliceto 80, 40100 Bologna, Italy*

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Abstract

An efficient and simple method for determination of methyl anthranilate (MA) in *Citrus* spp. honeys by headspace-solid-phase microextraction-gas chromatography (HS-SPME-GC) was developed and validated. Experimental design was used to investigate the effects of the principal extraction parameters. The central composite design (CCD) and the desirability function were used to find the experimental conditions providing the optimal HS-SPME result. Validation was carried out in terms of specificity, linearity, limit of detection (LOD) and quantitation (LOQ) (0.149 and 0.324 μg/g, respectively), method precision (RSD 7%), LOQ precision (RSD 6.5%), and was resulted accurate and robust. Finally, the applicability of the method to the determination of MA in a number of commercial *Citrus* spp. honey samples was demonstrated, the content ranged from 0.63 to 3.26 μg/g.

Keywords: Methyl anthranilate; Headspace-solid-phase microextraction; *Citrus* honey; GC; Experimental design; Method validation

1. Introduction

Honey is greatly appreciated by consumers as sweetener for its characteristic aroma, caused by the presence of many different volatile compounds. Honey aroma has been studied for years. More than 400 compounds have been identified and described as volatiles in honey of different floral types. However, it is expected that a large number of new volatile compounds will be identified in the future and there are still many honey types not yet successfully studied (Bouseta, Collin, & Dufour, 1992). Today, the characterization of flavour and quality control of monofloral honeys, *Citrus* spp. honey among them, is a subject of great interest in apiculture. The headspace-solid-phase microextraction-gas chromatography (HS-SPME-GC) technique can be successfully used for this purpose. This technique has been used for the analysis of volatile flavour compounds in honey. In 2001, Verzera et al. verified the possibility of characterization of Sicilian honeys from their qualitative volatile composition obtained by the SPME (Verzera, Campisi, Zappalà, & Bonaccorsi, 2001). Differences in the volatile fraction from several unifloral Spanish honeys recently observed by Pèrez et al. showed that, although the SPME technique can be used to determine the main volatile components of various Spanish unifloral honeys (orange, eucalyptus, rosemary, lavender, thyme) to characterize the floral source, further studies including others honey types are necessary (Perez, Sanchez–Brunete, Calvo, & Tadeo, 2002). Alissandrakis, Tarantillis, Harizanis, and Polissiou (2005), Baroni et al. (2006), Cuevas-Glory, Pino, Santiago, and Sauri-Duch (2007), De la Fuente, Martinez-Castro, and Sanz (2005), Odeh et al. (2007), Piasenzotto, Gracco, and Conte (2003), Soria, Gonzalez, De Lorenzo, Martinez-Castro, and Sanz (2004), Soria, Martinez-Castro, and Sanz (2003) used the SPME to characterize the aroma of different botanical origin honeys.

Methyl anthranilate (MA), a characteristic aroma compound of C*itrus* spp. honeys, was proposed as an indicator

* Corresponding author. Tel.: +39 059 2055147; fax: +39 059 2055131.
E-mail address: bertelli.davide@unimore.it (D. Bertelli).

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of the honey quality by Serra Bonvehì in 1988. He analyzed several *Citrus* spp. honeys from Spain, all classified as monofloral based on specified physical, chemical and melissopalynological characteristics. The MA content of these samples ranged between 0.57 and 4.2 ppm and led the author to conclude that, in spite of the low pollen content of some of these, a minimum of 0.5 ppm of MA qualified a honey as monofloral *Citrus* spp. (Serra Bonvehì, 1988). In 1995 Serra Bonvehì improved his studies and concluded that the MA content of >1.5 ppm, determined by the technique originally described in 1988, was a characteristic of marketable Spanish *Citrus* spp. honey (SerraBonvehì & Ventura, 1995). White et al. applied a photometric method for the determination of MA after steam distillation from honey solutions and diazotization (White, 1966; White & Bryant, 1996). Also the HPLC was used to evaluate the MA content in honey (Nozal, Bernal, Toribio, Jimenez, & Martin, 2001; Vinas, Campillo, Hernandez Cordoba, & Candela, 1992).

The development of methods for the MA analysis in *Citrus* spp. honey could be of great significance, however data on the application of the HS-SPME for the determination of MA are few in the literature.

In any experimental procedure, several experimental variables or factors may influence the result. A screening experiment is performed in order to determine the experimental variables and interactions which have significant influence on the result, measured in one or several responses. Therefore, the experimental design is taken for the optimization of the process parameters. The method developed and optimized is subsequently subjected to the validation.

Method development and optimization require the exploration of a space defined by different experimental parameters which are changed during experimentation in order to produce desirable values of the response. To achieve this aim, the experimental design selects the best location of experimental points in the predictor space and collects these points in a design matrix (Lundstedt et al., 1998).

Experimental design follows a sequential approach, it obtains information about the significance of the factors on the response then the information gained in the previous stage is used to decide which factors should be maintained and studied in later stages. As the study progresses, it is possible to adjust their variation range to a more promising region which can be explored more thoroughly. Moreover, it enables the effects of several variables to be simultaneously estimated (Pinzauti, Gratteri, Furlanetto, Mura, & Dreassi et al., 1996).

In the present work, a sensitive method based on the HS-SPME-GC was proposed for the MA determination in *Citrus* spp. honeys, the method development was conducted by a preliminary experimental design step using central composite design and response surface methodology to study the effect of the principal extraction variables (equilibrium time, extraction temperature and extraction time) on the analytical parameters used as response variables (peak area, peak resolution and peak width at half height) for MA determination. This response surface is approximated by a polynomial function and represents a good description of the relationship between the experimental variables and the responses within a limited experimental domain. The model generated contains quadratic terms which explain the non-linear nature of responses and multiple factor terms which explain the interaction effects between factors. When the number of responses is more than one, a composite response surface study is necessary to find the best compromise between the responses this study gives the co-ordinates of the optimum which allows the analysis of the substance to be performed. One of the techniques used to optimize several responses simultaneously is to weigh them together into one single criterion, which is desirability function (D). An overall D can be defined as a geometric mean of all individual desirabilities:

$$D = (d_1 * d_2 * \ldots d_n)^{1/n}$$

A calculation algorithm is then applied to D in order to determine the values of variables that maximize it. This set of variables is known as the "optimal point" (Pinzauti, Gratteri, Furlanetto, Mura & Dreassi et al., 1996). The developed and optimized method has to be validated. In fact, the ability to provide timely, accurate, and reliable data is central to the role of analytical chemists and is especially true in the development of analytical method. The method validation can be defined as the process of proving that an analytical method is acceptable for its intended purpose and in general, must include studies on linearity, detection limit, quantitation limit, precision, accuracy and robustness.

2. Experimental

2.1. Reagents and standards

Standard of methyl anthranilate (≥98%, purity), ethanol analytical grade and sodium chloride analytical grade were purchased from Fluka (Buchs, Switzerland).

2.2. Honey samples

The *Citrus* spp. honey used for the optimization and the validation procedures was provided by CRA–INA (Council for Research in Agriculture–Italian National Institute of Apiculture) (Bologna, Italy). The floral authenticity of this honey was confirmed by the same institute by melissopalynological and organoleptic analysis. For the validation procedure, a *Citrus* spp. honey with a low content of MA and a robinia honey (*Robinia pseudoacacia* L.), used as the blank sample, provided and certified by CRA–INA, were also used. Beside, eleven commercial Italian *Citrus* spp. honey samples were purchased on local market.

2.3. Software

Data acquisition and integration were made by using the software DataApex-Clarity™ 2.2.0.67 (DataApex Ltd, Prague, Czech Republic) for Windows.

Experimental design, data analysis and desirability function calculations were performed by using the software STATISTICA 6.1 for WINDOWS (Statsoft Inc., Tulsa, OK, USA).

2.4. Experimental design

To perform the experimental design, the equilibrium time (t_{eq}, min), the extraction temperature (T, °C) and the extraction time (t_{ext}, min) were selected as the experimental variables. The peak area (A), the peak resolution (R_s) and the peak width at half height (W_h) were selected as the quantitative and qualitative response variables. As well known, peak width and resolution, would be expected to depend principally on injection and separation conditions, nevertheless, these two parameters were introduce in the study to maximize the quality of the results. The factor levels and the experimental domain selected are shown in Table 1.

The next step in the planning procedure is to choose an experimental design. In this work, the optimization of the HS-SPME conditions was performed by the use of the central composite design (CCD, with $\alpha = 1.682$), based on a 2^3 full factorial design, plus six axial points, plus six replicates in the centre of the domain. The experiments were performed in triplicate and the resulting 48 experiments were conducted in randomized order. The analysis of the CCD results was performed by response surface methodology using a quadratic model, at last the measured responses were combined in one desirability function, after computing individual desirabilities.

2.5. HS-SPME procedure

For the analysis of MA content of honey samples, a 50/30 μm DVB/Carboxen™/PDMS StableFlex™ fiber was used. The fiber coating type was chosen because of its affinity to bipolar volatiles and its high sensitivity for smaller molecular compounds (Shirey, 1999). The fiber and the manual SPME holder were purchased from Supelco (Bellefonte, PA, USA). Before the HS-SPME-GC analysis, the fiber was conditioned in the injector of the GC system, according to the instructions provided by the manufacturer (60 min at 270 °C).

To 6 g of honey, exactly weighed in a 15 ml screw top amber vial, 2 ml of a saturated solution of sodium chloride were added (Yang & Peppard, 1994), then the vial was hermetically sealed with a polypropylene hole cap and PTFE/red rubber septa (Supelco, Bellefonte, PA, USA). Each sample was magnetically stirred and equilibrated during an equilibrium time in a thermostatic water bath at a desired extraction temperature (depending on the experimental design, Table 2).

Then, the SPME fiber was insert into the sealed vial by manually penetrating the septum and was exposed to the honey sample headspace during an extraction time (depending on the experimental design, Table 2). After the sampling, the SPME fiber was immediately insert into the GC injector and thermally desorbed.

Table 1
Factor levels and experimental domain selected for CCD

Experimental variables	Experimental domain				
	$-\alpha$[a,b]	−1	0	1	α[a,b]
Equilibrium time (t_{eq}, min)	1	16	38	60	75
Extraction temperature (T, °C)	40	50	65	80	90
Extraction time (t_{ext}, min)	6.5	20	40	60	74

[a] $\alpha = 1.682$.
[b] When necessary the levels with decimal digits were approximated to the nearest usable value.

2.6. GC analysis

A DANI 86.10 gas chromatography system equipped with an HP-5 (25 m * 0.2 mm I.D., 0.5 μm d_f) capillary column (J&W Scientific, USA) and a flame ionization detector (FID) system was employed. To optimize the GC conditions several injection temperatures and temperature programs were been tested. The best results were obtained using the following program, the column temperature was initially maintained at 50 °C for 4 min after injection, increased at 8 °C/min to 200 °C, held for 30 s, then programmed at 20 °C/min to 230 °C and kept for 1 min. Injections were done using the splitless system: the split valve was opened 5 min after injection. Helium at 1.5 bar was used as carrier gas. Injector and detector were maintained at 250 °C. A desorption time of 10 min at 250 °C was used.

2.7. Validation procedure

The optimized method was validated in terms of specificity, linearity, detection and quantitation limits, precision, accuracy and robustness.

Specificity of the analytical method was evaluated performing the GC–MS with the same chromatographic column and temperature program used for the GC analyses and evaluating the purity of the MA peak.

Linearity was established constructing a calibration curve with standards at the concentration of 0.6, 1.1, 1.7, 2.3, 3.4, and 5.7 μg MA/g of honey. Standards were prepared adding different volumes of a MA solution in ethanol (concentration of 1 μg/μl) directly in the blank honey.

Detection (LOD) and quantitation (LOQ) limits were calculated following the IUPAC approach which consists in analyzing the blank sample, calculating the standard deviation and expressing the result as the mean plus 3 and 10 times standard deviation for LOD and LOQ,

Table 2
The experiments proposed by CCD and responses obtained for MA

Experiments	t_{eq} (min)	T (°C)	t_{exp} (min)	A	R_s	W_h
1	60	80	60	1162.91	0.955	0.080
2	60	80	60	1109.21	0.979	0.077
3	60	80	60	1103.19	0.885	0.073
4	60	80	20	562.72	1.111	0.060
5	60	80	20	707.59	0.921	0.053
6	60	80	20	578.79	1.111	0.057
7	60	50	60	185.80	1.217	0.053
8	60	50	60	222.44	1.256	0.053
9	60	50	60	212.63	1.254	0.050
10	60	50	20	97.34	1.079	0.057
11	60	50	20	95.87	1.180	0.053
12	60	50	20	111.15	0.965	0.060
13	16	80	60	1121.93	1.122	0.073
14	16	80	60	865.05	1.180	0.063
15	16	80	60	853.57	1.242	0.067
16	16	80	20	346.01	1.370	0.053
17	16	80	20	540.63	1.287	0.057
18	16	80	20	503.48	1.215	0.060
19	16	50	60	212.95	1.561	0.053
20	16	50	60	224.28	1.337	0.053
21	16	50	60	192.53	1.217	0.053
22	16	50	20	92.30	1.079	0.060
23	16	50	20	80.69	1.111	0.053
24	16	50	20	94.07	1.180	0.057
25	75	65	40	450.99	1.145	0.060
26	75	65	40	531.94	1.249	0.057
27	75	65	40	460.93	1.217	0.053
28	1	65	40	386.17	1.252	0.052
29	1	65	40	307.30	1.125	0.057
30	1	65	40	400.45	1.475	0.060
31	38	90	40	1135.59	0.878	0.080
32	38	90	40	1024.65	0.855	0.073
33	38	90	40	1254.84	0.744	0.077
34	38	40	40	82.92	1.180	0.057
35	38	40	40	80.87	1.180	0.057
36	38	40	40	91.20	1.180	0.053
37	38	65	74	630.94	1.180	0.060
38	38	65	74	823.35	1.045	0.063
39	38	65	74	681.30	1.213	0.063
40	38	65	6.5	100.91	1.180	0.057
41	38	65	6.5	121.15	1.180	0.053
42	38	65	6.5	133.79	1.180	0.053
43	38	65	40	592.87	1.180	0.060
44	38	65	40	398.98	1.294	0.053
45	38	65	40	363.66	1.218	0.053
46	38	65	40	404.79	1.216	0.057
47	38	65	40	450.26	1.252	0.053
48	38	65	40	407.31	1.256	0.053

respectively (IUPAC Compendium of Chemical Terminology, 1997). In alternative, LOD and LOQ were calculated using the upper and the lower confidence limits of the calibration curve (H–V DLs) as reported by Hubaux and Vos (1970), with a confidence level of 95%. The H–V DLs can be determined graphically from a plot of the lower and upper prediction limits for the regression line in question.

Precision was evaluated through 24 repeatability analyses of the *Citrus* spp. honey sample estimated over three days. It was also evaluated the LOQ precision through 24 repeatability analyses of the blank robinia honey (*R. pseudoacacia* L.) added of a MA solution in ethanol (concentration of 1 μg/μl), in order to obtain a concentration of about the LOQ level.

Considering the impossibility to find a certified standard material and the lack of a reference official method for MA determination in honey, to evaluate the accuracy it should be used the recovery method. In our study the calibration curve was obtained spiking known amounts of the MA standard solution to sample matrix, in these conditions the recovery method loses some significance. Nevertheless different samples at three levels of reinforcement (0.40, 0.80, and 2.50 μg/g) were prepared using as reference the *Citrus* spp. honey sample with a low MA content provided by CRA–INA, this allows us to estimate the reproducibility of the extraction with honey at different level of MA content. All the analyses were performed five times.

Robustness was evaluated by an experimental design, performing small changes of the critical conditions (±2 min for t_{eq}, ±3 °C for T and ±2 min for t_{ext}) simulating the effect of random errors, and applying these conditions to the *Citrus* spp. honey sample. The number of the experiments was established by central composite design with $\alpha = 1.682$, all the analyses were performed in duplicate and 30 analyses were conducted in all. The results were compared by ANOVA with those obtained during the precision study.

2.8. Analyses of commercial Citrus spp. honey samples

The developed and validated method was applied to commercial samples to obtain some initial information on the MA content of Italian *Citrus* spp. honey and to asses the method applicability. All samples were purchased and analyzed in triplicate.

3. Results and discussion

3.1. HS-SPME optimization

The experimental conditions and the response values of the CCD are reported in Table 2.

The ANOVA of the obtained data (level of significance set at 5% ($P < 0.05$)) allows to evaluate the statistical significance of each factor and the interactions between the different factors. The T and the interaction between T and t_{ext} were the most significant parameters, having a strong positive influence for all the response variables. The effect of temperature can be accounted for in that it can influence the partition coefficient of the MA both between the sample and the headspace and between the headspace and the fiber, as well as the change in the vapour pressure of the MA in the sample.

For the A, the t_{ext}, the t_{eq} and the interactions between t_{eq} and T are also significant parameters. Instead, for the R_s, the t_{eq} also reaches the statistical significance, and about the W_h, the t_{ext} also results significant. Through the response surface study, it is possible to identify an optimal point only for the W_h variable.

To optimize the responses simultaneously, it was used a desirability function study. The individual desirabilities were assigned to each response variable with the purpose to obtain the wider A as possible, considering that, in general, the higher is the A the worse are the R_s and the W_h. For each response variable, it was assigned the individual desirabilities reported in Fig. 1. The desirability function allows to obtain as optimum setting the following experimental parameters: $t_{eq} = 19.5$ min, $T = 65$ °C, $t_{ext} = 56.82$ min approximated to 57 min. Fig. 2 shows the desirability surface plot for several combinations of the three experimental variables chosen. The optimum values were selected to evaluate the MA content of *Citrus* spp. honey samples in all the subsequent analyses.

3.2. Method validation

The specificity study shows that the peak purity measured by GC–MS is 98.8%.

The calibration curve equation is $A = 269.18 * \mathrm{MA} + 1.9781$ with $n = 18$ on six concentration levels. The value

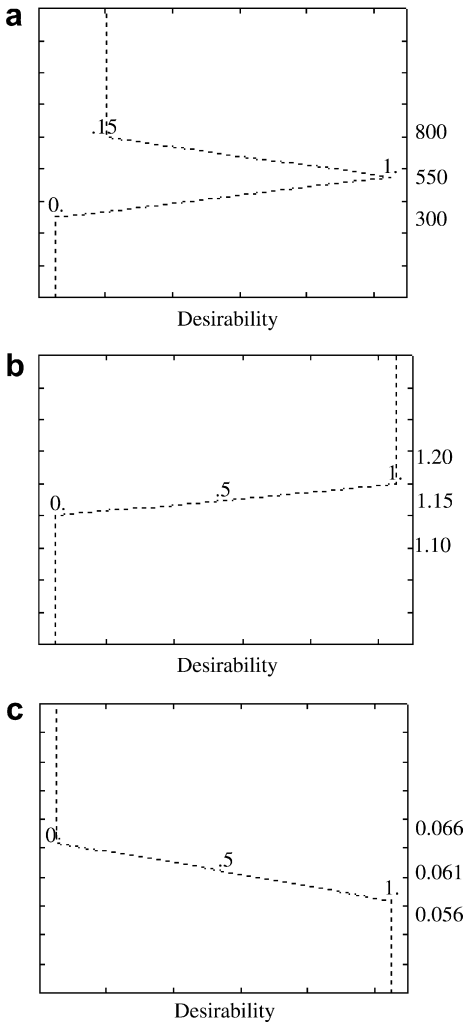

Fig. 1. Individual desirabilities for: (a) peak area; (b) peak resolution and (c) peak width at half height.

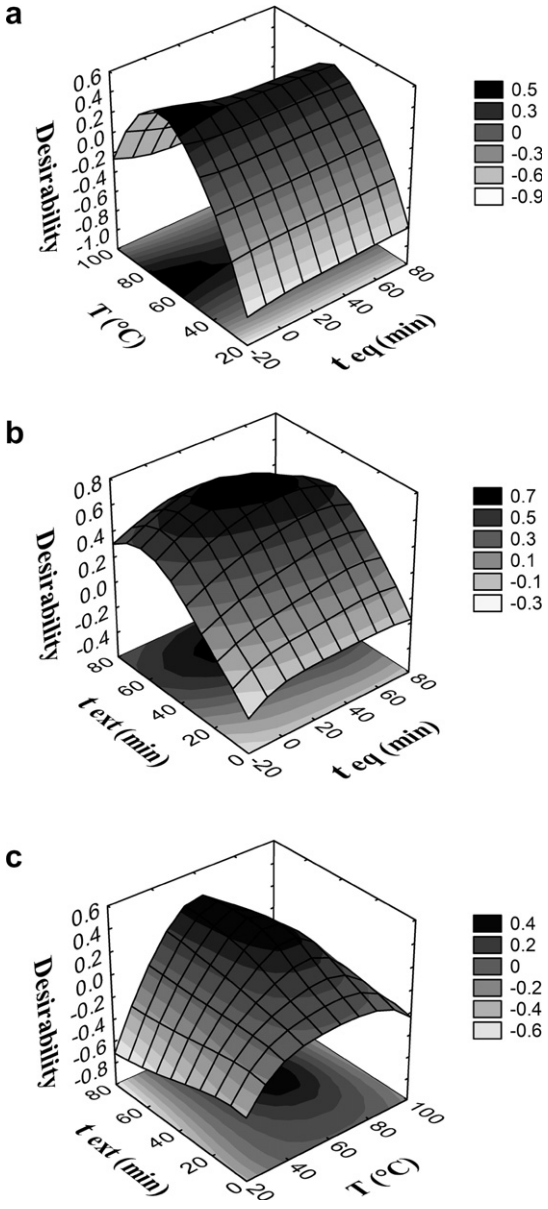

Fig. 2. Response surface plot for: (a) desirability versus extraction temperature (T, °C) and equilibrium time (t_{eq}, min), with a constant extraction time; (b) desirability versus extraction time (t_{ext}, min) and equilibrium time (t_{eq}, min), with a constant extraction temperature and (c) desirability versus extraction time (t_{ext}, min) and extraction temperature (T, °C), with a constant equilibrium time.

of the coefficient of determination ($r^2 = 0.9936$) indicates linearity of the calibration curve for the method in all the analytical range; this regression results statistically significant for $P < 0.05$.

The LOD values obtained using the IUPAC approach and the H–V DLs method are 0.149 and 0.138 μg/g, respectively and LOQ values 0.324 and 0.258 μg/g, considering more preferable to use the more prudential values, the ones obtained by IUPAC approach are selected as validation parameters.

The method precision and the LOQ precision show a relative standard deviation (RSD%) for the A values lower

than 7% and 6.5%, respectively and they could be considered good results for the HS-SPME-GC method applied.

Extraction recoveries of 99.0%, 103.6% and 102.9% are calculated for the samples spiked at 0.40, 0.80 and 2.50 μg/g of MA, respectively. These results are acceptable because the measured deviations are within ±7%, that is the RSD% obtained during the precision study, moreover considering that no statistical significant differences were found between the CV% of samples at different level of enrichment (3.1%, 2.8% and 2.8%, respectively) for $P < 0.05$, we can affirm that the proposed method shows an acceptable reproducibility at different level of MA content.

For the robustness, the obtained data are evaluated by ANOVA; this test shows that small changes applied to the experimental parameters do not cause significantly different results from those obtained in the precision study, confirming the robustness of the developed method.

3.3. Analyses of commercial Citrus spp. honey samples

A total of 11 samples of commercial *Citrus* spp. honeys were analyzed. Table 3 shows the MA content of each sample: the concentration range is between 0.63 and 3.26 μg/g. Samples 4, 7, 8, 10, 11 show a concentration higher then 1.5 μg/g, the minimum MA content characteristic of marketable Spanish *Citrus* spp. honey proposed by Serra Bonvehì (SerraBonvehì & Ventura, 1995). The other samples have a fewer MA content. In Fig. 3 a representative chromatogram of a commercial *Citrus* spp. honey sample is presented.

Table 3
MA content in *Citrus* spp. honeys of Italian origin

Samples	MA (μg/g) ± SD
1	0.72 ± 0.015
2	0.76 ± 0.028
3	1.19 ± 0.037
4	1.62 ± 0.085
5	1.17 ± 0.150
6	0.89 ± 0.037
7	3.26 ± 0.093
8	1.61 ± 0.083
9	0.63 ± 0.120
10	2.08 ± 0.036
11	1.61 ± 0.057

4. Conclusion

A method, which appeared to be sufficiently simple and fast, was developed and validated for extraction and determination of the MA content in *Citrus* spp. honey samples. The analytical approach was based on the use of HS-SPME-GC analysis. The use of an appropriate experimental design through desirability function study allowed to optimize the responses simultaneously and to calculate the optimal experimental conditions. These conditions were found in correspondence with t_{eq} of 19.5 min, T of 65 °C and t_{exp} of 57 min. Under these experimental setting values, the method was validated and it proved to be sensitive, precise, accurate and robust. The results obtained show that HS-SPME-GC can be successfully used in the analysis of MA in honeys in alternative to other approaches present in literature (Nozal et al., 2001; Vinas et al., 1992; White, 1966; White et al., 1996) and indicate that it should be a useful tool for the authentication of the floral origin of honeys. As also showed by many authors, the HS-SPME-GC is a reliable, reproducible and sensible technique with the advantage of being a simple, solvent-free and relatively economic fractionation technique (Alissandrakis et al., 2005; Cuevas-Glory et al., 2007). Therefore, this technique seems to be very promising in the analysis of volatile components of honey, although it is necessary to improve the studies before it becomes a widely usable technique. The method developed was successfully applied to analyse several commercial *Citrus* spp. honey samples of Italian origin, thus revealing different concentrations ranging from 0.63 to 3.26 μg/g and, as evident, this range is wider than the results obtained by others authors in Spanish *Citrus* spp. honeys (Ferreres, Giner, & Tomas-Barberan, 1994; Nozal et al., 2001). In the next future, it could be of great interest to make further researches to identify, if it should exist, a more reliable level

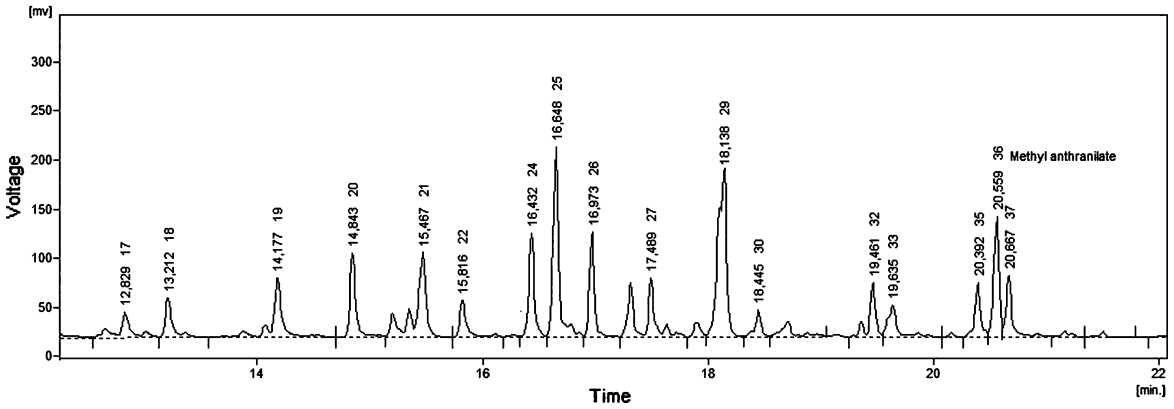

Fig. 3. Representative chromatogram of *Citrus* spp. honey (sample 4); MA retention time: 20.56 min. The sample contains 1.56 μg MA/g.

of MA content usable to identify Italian *Citrus* spp. honey from different geographical areas.

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