In tube extraction for determination of the main volatile compounds in *Physalis peruviana* L.

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Abbreviations: CIM, conventional interpolative method; **EI**, electron impact; **GC×GC**, two dimensional gas chromatography, **HS**, headspace; **ITEX**, in tube extraction;

Abstract

An analytical procedure based on in-tube extraction followed by gas chromatography mass spectrometry has been developed for the analysis of 24 main volatile components in cape gooseberry (*Physalis peruviana* L.) samples. According to their chemical structure, the compounds were organised in different groups: 1 hydrocarbon, 1 aldehyde, 4 alcohols, 4 esters and 14 monoterpenes. By single-factor experiments, incubation temperature, incubation time, extraction volume, extraction strokes, extraction speed, desorption temperature and desorption speed were determined as 60 ^{0}C, 20 min, 1000 μL, 20, 50/50 μL/s, 280 ^{0}C, 100 μL/s, respectively. Quantitative analysis using authentic standards and external calibration curves was performed. The limit of detection and limit of quantification for the analytical procedure were calculated. Results shown the benzaldehyde, ethyl butanoate, 2-methyl-1-butanol, 1-hexanol, 1-butanol, α-terpineol, terpinen-4-ol were the most abundant volatile compounds in analysed fruits (68.6 - 585 μg/kg). The obtained data may contribute to qualify cape gooseberry to group of superfruits and therefore increase its popularity.

Keywords

in-tube extraction; gas chromatography; cape gooseberry; fruit; terpenes

1. Introduction

The word “superfood” has been recently introduced to the nomenclature [1]. It comprises 14 natural products among which can be found e.g. fruits, vegetables, corns and tea. These food ingredients introduced into human diet bring many health benefits and can easily enhance well-being. A large group of nutrient-rich fruits played an important role in folk medicine in Asia (China, Tibet) and Africa for thousands of years. Nowadays, the “superfruit” is treated more like a marketing term than a science and that is the reason why food and medicinal preparations based on these kind of fruits are more and more popular among consumers. The globalization of world markets enables the availability of even the most exotic fruits which can be used in order to enrich the diet with new flavours while providing many significant health natural ingredients [2]. The term superfruits is considered as a new marketing approach to promote the demand for rare fruits which can be consumed as foodstuffs or used as ingredients by manufacturers of functional foods, nutraceuticals, beverages. However, gaining the popularity of health-oriented, superfruits on market depend heavily on both research results and appropriate marketing. Fruits which contain powerful bioactive compounds such as polyphenols, anthocyanins or procyanidins, with high antioxidant capacity may be classified as a superfruits. Also very important is contents of terpenes, because they determine the flavour and taste of fruits and many of them have bioactive properties e.g. α-phellandrene and β-myrcene has antioxidant properties [3], limonene has antimicrobial, antidiabetic, antifungal [4-7], *p*-cymene has antibacterial, antinociceptive and anti-inflammatory [8-10] properties. Considerable interest led to the increase of the number of research and publications focusing on health benefits of superfruits [11-14] and determination terpenes compounds in food products [15-17].

Physalis peruviana, commonly known as goldenberry or cape gooseberry, is a solanaceous hairy plant native to tropical South America. Cape gooseberry is an herbaceous, semi-shrub,

upright and perennial growing in subtropical zones. Its general size is between 0.6 to 0.9 m but in some cases it can reach 1.8 m. The flower can be easily pollinated by insects, wind and also by auto-pollination. The fruit is a juicy berry with ovoid shape and a diameter between 1.25 cm to 2.50 cm, 4 g and 10 g weight, containing inside around 100 to 200 small seeds [18]. The cape gooseberry is extensively used as medicinal herb for treating diseases such as cancer, malaria, asthma, hepatitis, dermatitis and rheumatism [19]. There are known additional attributed properties such as antispasmodic, diuretic, antiseptic, sedative, analgesic, helping to fortify the optic nerve, throat trouble relief, elimination of intestinal parasites and amoeba. There have also been reported antidiabetic properties, recommending the consumption of five fruits per day. There are studies indicating that eating the fruit of cape gooseberry reduces blood glucose after 90 min postprandial in young adults, causing a greater hypoglycaemic effect after this period [20]. So far, there are no studies that indicate possible adverse effects. Cape gooseberry is an attractive fruit for international markets due to its important nutritional as well as medicinal properties. Currently, there are different products made of this fruit such as jams, raisins and chocolate-covered candies. It can also be processed for juice, pomace and other products sweetened with sugar as a snack [21]. However, it is still one of the less consumed raw materials of plant origin for human nutrition.

In-tube extraction (ITEX) combines efficient sample extraction, with selective analytes concentration and rapid transfer to GC-MS system. A micro trap filled with adsorbent materials is placed between the HS syringe and a needle. This allows a rapid, simple and efficient extraction and concentration of volatile compounds. Analysis is carried out by multiple pumping of headspace fraction in the closed vial through adsorbent located in a special type of needle [22]. The main advantages of the in-tube extraction are: i) its effectiveness with highly volatile compounds, ii) the possibility of optimising its

concentration capability, depending on the analytes amount in the vapour phase by selecting a suitable number of pull/push cycles [23, 24].

The aim of the present work was to identify main volatile compounds from *Physalis peruviana* and optimize ITEX extraction method for their subsequent quantitation by GC-MS.

To our knowledge there are no reports on use of the ITEX for analysis of gooseberries volatile compounds. Previous publications on determination of volatile compounds in that fruit were related to liquid-liquid extraction [25, 26], dynamic headspace [27] and solid phase microextraction techniques [28-30]. Only in two publications information on quantitative data of few compounds [26, 29] were reported, as well as semiquantative using of relative percent area [27, 28].

2. Materials and Methods

2.1. Materials

All standard chemicals: α-pinene ≥99%; β-pinene ≥99%; limonene ≥99%; ocimene ≥90%; γ-terpinene ≥97%; α-terpineol 97%; β-citronellol ≥99%; β-myrcene ≥90%; *p*-cymene 99%; eucalyptol ≥99%; α-terpinolene ≥90%; terpinen-4-ol ≥95%; α-phellandrene ≥95%; geraniol ≥99%; ethyl butanoate ≥99.5%; butyl acetate ≥99.7%; ethyl octanoate ≥99%; ethyl decanoate ≥99%; 1-butanol ≥99.9%; 1-hexanol ≥99.9%; heptan-2-ol ≥97%; *n*-pentanal ≥97.5%; 2-methyl-1-butanol ≥99%; benzaldehyde ≥99%; were purchased from Sigma-Aldrich (Sigma-Aldrich, Poznań, Poland).

Samples of cape gooseberry (*Physalis Peruviana* L.) imported from Colombia and purchased at supermarket were analysed. Prior to analysis, fruit samples (calyx removed) were stored in the freezer at -35 ^{0}C.

2.2. Methods

2.2.1. Sample preparation

Before the extraction step, the fruits were pureed using a mortar and pestle. NaCl was added (10% *w*/*w*) during the blending stage in order to prevent possible enzymatic reactions that can lead to the conversion of some volatile compounds to their derivatives and to increase the concentration of analytes in the sample headspace [31]. The fruits reached the room temperature before proceeding with the ITEX extraction. Eight grams of sample were moved to 20 mL vial crimped with Teflon coated silicon rubber septa.

2.2.2. Optimized extraction procedure

The extraction process was carried out with a commercial version of ITEX installed in autosampler (Alpha M.O.S. HS100) with PAL1 Cycle Composer software (version 1.5.4). A 2.5 mL headspace ITEX syringe (Hamilton Bonaduz AG, CTC Analytics, Switzerland) was used with the ITEX trap (Tenax TA 80/100 mesh). The ITEX extraction parameters were a subject of study, the optimal parameters have been provided in Table 1.

2.2.3. Instrumentation

The analysis was carried out on an Agilent 7890A gas chromatograph with single quadrupole mass detector (Agilent Technologies, 5975C VL MSD, (TAD)). The injector was a standard split/splitless. The injection was carried out in a split mode (1:10). The carrier gas was He at a constant linear velocity of 32.4 cm/sec (pressure 15.7 psi, flow of 0.8 mL/min) during the run. The column was DB-5 (Agilent Technologies, 30 m × 0.2 mm I.D., 0.2 μm film thickness). The chromatographic oven was held at 40 ^{0}C for 1 min, then raised to 200 ^{0}C at 10 ^{0}C/min, then to 280 ^{0}C at 20 ^{0}C/min and finally the temperature was held at 280 ^{0}C for 1 min. Analyses were performed in electron impact (EI) mode. The ion source temperature was 230 ^{0}C GC/MS interface was kept at 280 ^{0}C. Detection was in a scan mode with *m/z* 33 to 333 range. The ITEX/GC-MS process was carried out according to optimized conditions.

2.2.4. Data analysis

Tentative identification was accomplished through MS library search using the NIST (version 2.0) mass spectral library. Positive identification of 24 analytes (α-pinene, β-pinene, limonene, ocimene, γ-terpinene, α-terpineol, β-citronellol, β-myrcene, p-cymene, eucalyptol, α-terpinolene, terpinen-4-ol, α-phellandrene, geraniol, ethyl butanoate, butyl acetate, ethyl octanoate, ethyl decanoate, 1-butanol, 1-hexanol, heptan-2-ol, *n*-pentanal, 2-methyl-1-butanol, benzaldehyde) was confirmed by the comparison of retention times with authentic standards. Moreover, an ITEX blank run was done every one analysis of fruit samples as well as standards to consider the influence of column or Tenax degradation. The analysis of fruits sample was performed in five repetition. The calculation were performed using Excel 2010, Microsoft Office 2010. In order to define significance of differences the statistical tests were used (the Fisher-Snedecor test, the Student's t-test, the c-Cochran and Cox test).

3. Results and discussion

3.1. Optimization of extraction conditions

To provide the highest peak responses and best resolution of analysed compounds the following parameters were optimized for the ITEX extraction: incubation temperature, incubation time, extraction volume, extraction strokes, extraction speed, desorption temperature and desorption time. For the extraction method optimization, a mixture of 12 compounds detected in cape gooseberry was used and peak areas were compared in these experiments. The following compounds were chosen to represent main classes of volatiles present in gooseberries: alcohols (2-methyl-1-butanol and 1-hexanol), esters (butyl acetate and ethyl octanoate), monoterpene hydrocarbons (β-myrcene and α-terpinolene), monoterpene alcohols (terpinen-4-ol and α-terpineol), monoterpene aromatic hydrocarbon (*p*-cymene), monoterpene cyclic ether (eucalyptol), aromatic and aliphatic aldehydes (benzaldehyde and pentanal). The average dry matter of cape gooseberry is 20,7 % (*w/w*). The vast majority of the fruit consists of water, therefore the water standards solutions was used to select optimal

parameters of the extraction process. The repeatability of the extraction under tested conditions were calculated as relative standard deviation of absolute peak areas for the triplicate analyses of model samples. Table 1 presents summarized optimization parameters and tested values.

3.1.1. Effect of incubation temperature and incubation time

In the sample analysis via ITEX, analytes are extracted from the sample headspace. Therefore, the temperature and the time at which equilibrium is reached between the concentration of analytes in the sample and the sample headspace are crucial parameters. Figure 1A and Figure 1B show the results of analyses performed in order to optimize the temperature and time of extraction steps.

All the extraction temperatures were tested at the same extraction time of 10 minutes. The highest extraction efficiency for all compounds was noted at 60 ^{0}C. The extraction efficiency increased along with an increase of the extraction (incubation) temperature. It is known that in the higher temperatures thermal degradation of compounds can take place. Therefore, the extraction temperature was established at 60 ^{0}C and it was used for subsequent analyses.

The next step involved optimization of the extraction time. In case of five compounds (β-myrcene, α-terpinolene, p-cymene, eucalyptol, benzaldehyde) the highest extraction efficiency was obtained at 5 min. For esters and monoterpene alcohols the most convenient time was 20 min. The lowest repeatability of the analysis was observed for 10 min of incubation. In case of alcohols, the difference in the extraction efficiency in different incubation time was not significant. Considering the above the optimal incubation time was established at 20 min.

3.1.2. Effect of extraction volume

The following volumes of extraction were tested: 300, 500, 1000, 2000 μL. For the 300 μL the significant problem with repeatability of the peaks area in subsequent analyses was observed. For almost all compounds the highest efficiency of extraction in the volume of 2000 μL were obtained (only for butyl acetate in 1000 μL peaks were the most abundant). In case of 500 μL for esters the lowest repeatability was observed. The results are presented in Figure 1C.

At first 2000 μL was chosen as the optimal extraction volume. However, in further analyses the leaks in syringe was observed (twice). Therefore, it was decided to choose 1000 μL as optimal volume (the leaks at syringe was not observed).

3.1.3. Effect of extraction strokes

The relationship between the number of extraction cycles and signal of analytes is presented in Figure 1D. For nine of the compounds with increasing number of strokes extraction efficiency increased. Only for monoterpene hydrocarbons and monoterpene aromatic hydrocarbon the maximum of extraction efficiency was obtained at 20 and 30 strokes, respectively. However, it should be noticed that increasing numbers of strokes increases the extraction time (in case of 40 strokes extraction time is 4 times longer than for 10 strokes, it gives 5 min and 20 min respectively, the test were carried out in 50 μL/s of aspirate speed and 100 μL/s of dispense speed). Moreover, the greater is the number of stokes the higher is the risk of the syringe leaks in subsequent analyses. Therefore, the optimum value as 20 strokes was chosen.

3.1.4. Effect of extraction speed

Extraction speed consists of aspirate and dispense speed. The first is related to the speed of rising the syringe plunger, the second with the speed of lowering the syringe plunger during extraction process. Figure 2A presents the relationship between the number of extraction

cycles and signal form analytes. An increase of the extraction (aspiration and dispense) speed lead to decrease of the extraction time. For the tested parameters the extraction times were as follows: 13.3 min (50/50), 10 min (50/100), 6.6 min (100/100), 4.4 min (100/300) and 2.2 min (300/300). However, the higher is the dispense speed the higher is pressure in syringe and the risk of the syringe leaks increases [32]. In case of all analysed monoterpene hydrocarbons, the extraction speed is decreasing with growing extraction speed. For the rest compounds the minimum is reach in 100/100 μL/s. Taking into account the above, the optimal extraction speed established 50/50 μL/s.

3.1.5. Effect of desorption temperature and desorption speed

In order to ensure quantitative transfer of the analysed compounds adsorbed on the ITEX trap to the chromatography system, an adequate trap temperature during desorption and speed of the desorption process is required. Both conditions should not promote the formation of artefacts and thermal degradation of the stationary phase of the ITEX sorbent, whereas the analytes should be completely desorbed from the Tenax TA.

The results of analyses carried out using various desorption temperature of analytes by the ITEX trap are present in Figure 2B. For esters, monoterpene hydrocarbons and monoterpene aromatic hydrocarbon, the most intense peaks were observed in desorption temperature of 240 ^{0}C, while the most reproducible results were obtained at 280 ^{0}C. For the remaining compounds (especially alcohols and monoterpene alcohols) no significant difference was observed.

The carry over effect was checked for all standards and was tested in different desorption temperatures. It was found that this effect occurs for four compounds (β-myrcene, p-cymene, eucalyptol, α-terpinolene). The results of analyzes carried out using various desorption temperatures from the Tenax TA are present in Table 2. In the desorption temperature of 240

234 ^{0}C carry over is more than 0.1% after the first analysis, moreover the highest is for β-
235 myrcene. The lowest carry over was achieved at 280 ^{0}C. The highest decline in the size of the
236 peaks area as the temperature increases was observed for β-myrcene (240 ^{0}C - 0.258%, 260
237 ^{0}C - 0.158%, 280^{0}C - 0.099%). Furthermore, the highest repeatability of the analyses was
238 achieved in the temperature of 280 ^{0}C. Therefore, the 280 ^{0}C of desorption temperature was
239 chosen as optimal for further analysis.

240 The results of analyses carried out using various desorption speed of analytes from the Tenax
241 TA are presented in Figure 2C. For all compounds the most intense peak areas at 100 μL/s
242 were observed, while for esters the smallest repeatability of the analyses. Also in the lowest
243 desorption speed the best peaks shapes (narrows and fully separated) was achieved.

244 Based on the literature data, for the volatile compounds determination (including terpenes) in
245 fruits samples the injector temperature of 250 ^{0}C and 3 min was found as optimum [33-35].

246 **3.1.6. Exhaustion extraction of analytes from the sample using in-tube extraction**

247 In order to check the number of analysis that can be performed on one sample, provides the
248 result reliable, it was carried out a exhaustion test of the sample. For the first two analyses no
249 significant decrease in total peak areas was observed (statistically significant). For subsequent
250 analyses decline in the total peak area with the number of repetitions was evident. The results
251 of analyses are presented in Figure 3.

252 **3.2. Performance of the analytical procedure**

253 The performance of the optimized analytical procedure for the analysis of 24 the most
254 concentrated volatile compounds in cape gooseberry fruit by ITEX/GC-MS was evaluated by
255 applying the extraction procedure as described in Table 1.

For the creation of standard curves, 8g of cape gooseberry (after isolation step using Dering apparatus) with salt addition were spiked with mix of standards in the range of concentrations. The analysis was performed by GC-MS using an external calibration curve method (CIM - Conventional Interpolative Method). Using this method several standard solutions of various, known concentrations of the analytes were prepared (in range 5 - 500 μg/kg). Making measurements for mix of standard solution and for sample, the calculations was done in the interpolative way (in the linearity range of calibration curves). The stock solutions of standards were prepared in methanol. For each standard the following concentrations were analysed: 5, 10, 25, 50, 100, 250, 500, μg/kg. Each of standard solution concentrations was run three times.

The LOD and LOQ for the analytical procedure were calculated on the basis on the standard deviation of a set of signals and the angle of inclination of the calibration curve. The equations of calibration curve in the range of linearity, linearity range, coefficient of determination, detection and quantification limits in order of retention time of analytes were shown in Table 3.

3.3. Analysis of compounds in cape gooseberry sample

The volatile compounds determined in cape gooseberry are presented in Table 4. Compounds with the highest peak areas (24) after first test analysis with use of ITEX/GC-MS were chosen. This group consisted of 1 hydrocarbon, 1 aldehyde, 4 alcohols, 4 esters and 14 monoterpenes.

19 of 24 compounds were previously identified by using LLE/GC-MS [25], this include 1 hydrocarbon, 1 aldehyde, 4 alcohols, 4 esters and 9 monoterpenes. 21 of 24 compounds were previously identified by using HS-SPME/GC-MS, this include 1 aldehyde, 4 alcohols, 4 esters and 12 monoterpenes [28] and additional 112 compounds were determined.

Moreover, the profile of volatile terpenes in cape gooseberry was determined using GC×GC-ToFMS. The 62 terpenes were identified [30], and it confirms 14 chosen monoterpenes in this work. The cape gooseberry is known for its high percentage content of compounds from the group of terpenes, compared to other fruits [29]. For this reason, it is assumed that cape gooseberry is starting to be known as superfruit.

The 1-butanol, 2-methyl-1-butanol, heptan-2-ol, 1-hexanol and α-terpineol were also reported by Mayorga et al. [36], with additional 39 compounds as a glycosidically bound flavour compounds. Also α-pinene, ethyl octanoate and eucalyptol (as 1,8-cineole) were previously determined by Ramadan et al. [27] with additional 31 compounds.

Previously reported quantitative results by Ymaztekin [26] for some of analytes are different comparing to data obtained in this paper (benzaldehyde 110.4 μg/kg, 1-butanol 514.3 μg/kg, heptan-2-ol 10.07 μg/kg, 1-hexanol 292.9 μg/kg, 2-methyl-1-butanol 470.4 μg/kg, butyl acetate 19.4 μg/kg, ethyl decanoate 130.2 μg/kg, ethyl octanoate 28.8 μg/kg, β-citronellol 26.0 μg/kg, terpinen-4-ol 128.5 μg/kg, α-terpineol 160.7 μg/kg, β-myrcene 7.9 μg/kg, ocimene 2.8 μg/kg, α-terpinolene 13.2 μg/kg). For determination of volatile compounds the liquid-liquid extraction with combination of GC-FID and GC-MS was used. However, for quantification only 4-nonanol, γ-valerolactone and cyclohexyl butanoate were used. Whereas Dymerski et al. [29] determined terpinen-4-ol (50 μg/kg), γ-terpinene (95 μg/kg) and α-terpinolene (180 μg/kg) using HS-SPME/GC×GC-ToFMS. The difference in the obtained results may be due to the biological sample, different origin, agronomic and climatic conditions, as also store the fruits during transportation.

The benzaldehyde, ethyl butanoate,1-hexanol, 2-methyl-1-butanol, 1-butanol, α-terpineol was found in greatest concentrations (more than 70 μg/kg). 1-butanol and 2-methyl-1-butanol have a sweet, floral and fruity notes [26]. This alcohols are reported in many exotic fruits as

acerola, jackfruit, *Annona cherimolia* or *Spondias mombin* [37]. α-Terpineol is known in antimicrobial effects [38].

From the state of the art it is known the Tenax is releasing aldehydes (e.g. benzaldehyde) and ketones during thermal desorption, which can obscure the determination of these compounds [32, 39]. However, in consideration of linearity and reproducibility of the content of benzaldehyde, it can be assumed that the impact of benzaldehyde derived from sorbent is not significant. It is certain that the benzaldehyde is present in the fruit, as indicated in the literature [25, 29].

As was observed during optimization of the ITEX/GC-MS method the concentration level was different for single compounds (very abundant peak of one compound, in fact, did not indicate a high concentration of this compound). It depends of the LOD and LOQ, the linearity of calibration curve, selectivity and sensitivity of GC system. This is related to all relative quantitative methods - they are not so accurate and reliable as quantification using authentic standards and calibration curves.

4. Concluding remarks

The method development for in-tube extraction and gas chromatography was successfully applied to the analysis of the volatile fractions from cape gooseberry fruit. The results indicate that the ITEX/GC-MS technique is a good alternative for the determination of volatile and semi-volatile compounds, in particular terpenes, compared with other concentration techniques and separation methods for volatile analytes. Also it limits the use of chemical reagents in the sample preparation step. The principal components analysis indicated that the *Physalis peruviana* L. is mainly composed of compounds from the branched esters, alcohols and monoterpene groups. Literature data about flavour compounds of cape gooseberry are rare. It is assumed the obtained data will contribute to qualify these fruits to group of

superfruits and also increase their popularity. The results of this research may encourage both the food and pharmaceutical industry to utilize these fruits as raw material or additives for new, health-oriented food products (such as fruity juices, wines and liqueurs) and nutraceuticals including dietary supplements. As a result, the human diet will be supplemented with additional healthy and valuable products.

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Conflict of interest

The authors have declared no conflict of interest.

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451 **Figure captions**

452 **Figure 1.** Optimization of the extraction parameters: (A) incubation temperature, (B)
453 incubation time, (C) extraction volume, and (D) extraction strokes by single factor-
454 experiments. The error bars based on triplicate analyses are included.

455 **Figure 2.** Optimization of the extraction parameters: (A) extraction speed, (B) desorption
456 temperature, and (C) desorption speed by single factor-experiments. The error bars showing
457 standard deviation based on triplicate analyses are included.

458 **Figure 3.** Exhaustive extraction for gooseberry sample performed from a single vial.