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# ANALYSIS OF FOOD SUPPLEMENT WITH UNUSUAL RASPBERRY KETONE CONTENT

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## ABSTRACT

In recent years food supplement market increased constantly, including slimming products and against obesity. The case of rasberry ketone (RK) is here reported. HPTLC and HPLC-DAD analyses on a marketed product containing raspberry juice evidenced an abnormal quantity of RK, not in accordance with the juice natural content. The reported data confirm the need of adequate controls on marketed food supplements and the necessity of a complete adherence between labelling and real constitution of the product.

## PRACTICAL APPLICATIONS

Determining the natural origin and assuring the consumers' safety for raspberry-based food supplements.

## INTRODUCTION

Obesity is reported as the second leading environmental cause of death in USA and the fifth of global deaths (James *et al.* 2001; Flegal *et al.* 2010; Ogden *et al.* 2014). According to the WHO definition, obesity is an abnormal or excessive fat accumulation that may impair health. Obesity is considered an epidemic phenomenon, interesting many parts of the world, and therefore it was named by the WHO with the suggestive term of Globesity (WHO/FAO 2002a,b; WHO 2008). If obesity is considered a disease, people look for a solution. The most simple solution should be changing and control of food input and life style, but often both the result very difficult to achieve. The other solution consists in the use of potentially effective medical measures to both prevent and treat obesity. Most of the synthetic drugs once in use are nowadays banned, not considering the difficulties to demonstrate the real weight loss in the long period. Also the remaining ones are pending or are going to be out of the official market. On the other side, natural products recently entered massively in the field in form of food supplements based on plant extracts or their constituents (Nicoletti 2012; El Sohaimy 2012).

An aggressive marketing and consumers expectative tend to assign several properties to food supplements, including miracles in weight loss. In the recent years, botanicals marketed for reducing fats and limiting obesity registered an evident boom, whose reality is difficult to determine, since most of the market is conveyed through Internet. The types of marketed products are continuously changing. Part of them, based on adrenergic and serotonergic activity, can be rationalized following a molecular pathway. Everything started with amphetamines, which dominated for a long time, with satisfaction of sailings and consumers. After the amphetamine ban, due to severity of side effects as most of the synthetic drugs, attention on weight reducing products shifted to natural products, exploring in particular those based on $C_6C_2N$ molecular model that means compounds with structures similar to amphetamines and inspired to those of dopamine, adrenaline and similar CNS activators (Fig. 1). All these compounds are biosynthetically derived from the aromatic amino acids phenylalanine/tyrosine.

The basic idea is that similarity in structure should lead to similarity in reaction on the active center of the receptor that could be accepted in general but not in specific. The situation is even complicated by the presence of mixtures of

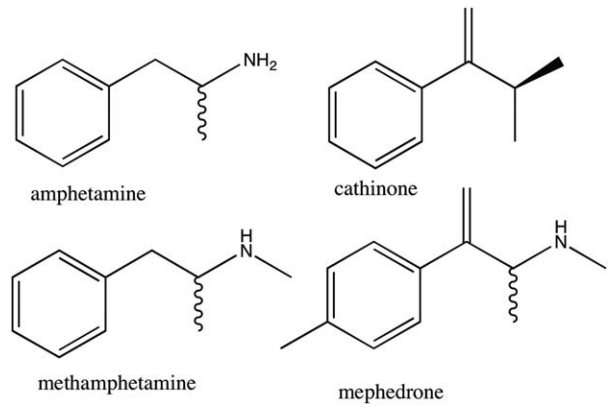


**FIG. 1.** STRUCTURAL CORRELATION BETWEEN C6C2N SUBSTANCES WITH ACTIVITY ON NERVOUS SYSTEM

various isomers. The relative sequence of amphetamine-like natural products comprehends ephedrines from *Ephedra sinica* and catines and catinone from *Catha edulis*, also banned for side effects, and synephrines from *Citrus* juices, under consideration for the limit of dosage.

Recently, attention was focused on several ketones, as reported by the press in the 2015 Tour the France. One of the last new entries in the weight-loss market, with related structure to $C_6C_2N$ model (Fig. 2) is the so-called raspberry ketone (RK). RK came under people attention, due to an impressive advertisement campaign in Internet and TV, focused on amazing slimming effects. However, first it is necessary to consider the name raspberry ketone concerns either several products based on raspberry juice and a definite compound under the IUPAC name 4-(*p*-hydroxyphenyl)butan-2-one that can be found in raspberry juices and in other fruits. In RK structure, the main sequence is maintained, but the amine function is substituted by the carbonyl. Actually, RK use is allowed in EU as flavouring agent (Regulation EC 1334 2008). In 2013, UK's Food Standards Agency (FSA) launched an alert on RK, warming firms that RK, in the use in food supplements, must be considered a novel food (Food Standard Agency 2013). In March 2014 raspberry ketone was classified by FSA as a novel food. It was stated that raspberry ketones other than raspberry fruit extracts prepared using water or 20% ethanol (1:4 ethanol:water) are novel and should fall within the scope of the EU legislation on novel foods (Food Standards Agency 2007). According to the EU legislation on food supplements (Regulation EC 268 1997), an ingredient is a novel food when there is no evidence of its consumption or ordinary production to a significant degree in EU before 15 May 1997. In case of a novel food, the interested company can present a dossier for the registration to commercialization, including the related claim. In fact, foods not known in European communities can be used and eaten for long time in other parts of the world. However, a health claim application for *Rubus idaeus* (raspberry) extract – BERI-08 – and thermogenesis production, satiety and consequently weight loss was rejected by the European Food Safety Authority (EFSA) due to the lack of evidence. Later, EFSA reinforced this approach, considering the product potentially unsafe. In any case, EU's Rapid Alert System for Food and Feed reported the circulation of RK products in Europe, as well as the consequent pick up by local authorities.

The major concern is on the content of food supplements. As a matter of fact, several botanicals are under accusation for the content in the active ingredient that could be much higher than the natural one, and therefore intentionally added to increase activity and performance of the product. The added substance, rising also up the 80%, could be of synthetic or natural origin. Therefore, analytic control of the content of suspicious marketed food supplements is necessary to avoid side effects. Analytic control must be tailored to the complex and particular nature of botanicals. In this paper, we report the analysis performed on the commercial product Raspberry Keton, found in the Italian pharmaceutical market, in order to determine the present quantities of RK and synephrines. Analyses were performed by HPLC-DAD and HPTLC (High Performance Thin Layer Chromatography) methods. Initially, HPTLC was used as a rapid and simple tool for evidencing all the complicated composition, derived from the mixture of several extracts (Nicoletti 2011; Gallo *et al.* 2012; Toniolo *et al.* 2013). HPLC-DAD allowed a quantitative determination of RK in the food supplement analysed.

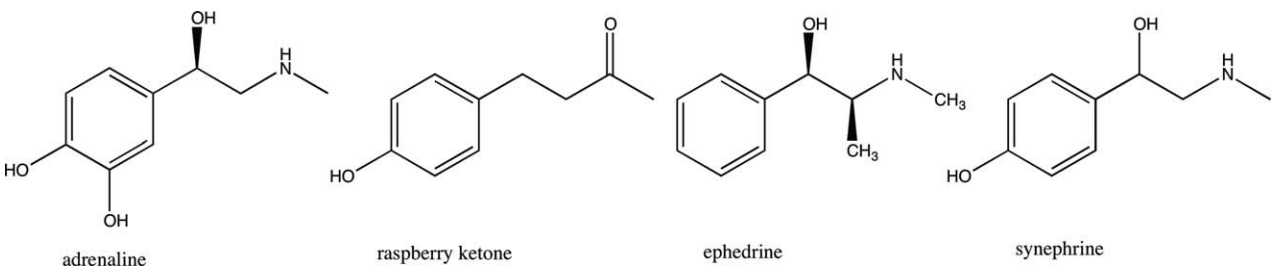


**FIG. 2.** STRUCTURAL CORRELATION BETWEEN C6C2N SUBSTANCES AND RASPBERRY KETONE

# MATERIALS AND METHODS

## Reagents and Materials

The analysed marketed product Raspberry Ketone 400 liquid reports in the label: content of several juices or extracts (Rasberry [*Rubus idaeus* L. *fructus*] 400 mg; orange fruit [*C. aurantium* L. sub. *amara fructus immaturus*] 160 mg with *p*-sinephrine accounting for 9.6 mg; onion [*Allium cepa* L.] 40 mg; chrome 40 μg; protein <0.2 g; lipids <0.2 g; carbohydrates 4.7 g. Use: 25 days, 20 mL/day). The analyses were performed in HPTLC on two samples (different batches), but their fingerprints resulting identical, thus HPLC analysis was performed only on one product. Solvents of RPE grade were purchased from Sigma–Aldrich (Milan, Italy) or Carlo Erba Reagenti (Milan, Italy), silica gel 60 (70–230 mesh ASTM) from Fluka (Milan, Italy). RK standard (99%) was obtained by Sigma–Aldrich. It was used in HPTLC analysis at 1 mg/mL concentration (Reagent Water System, Millipore, Bedford, MA). Disposable Minisart SRP4 filters with a pore width of 0.45 μm (Chromafil PET-20/25) were from Sartorius Stedim Biotech GmbH (Goettingn, Germany).

## HPTLC Analysis

**Instrument.** The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of Linomat 5 sample applicator using 100 μL syringes and connected to a nitrogen tank; automatic developing chamber ADC 2 containing twin trough chamber 20 × 10 cm; Immersion device III; TLC Plate Heater III; TLC visualize linked to winCATS software. Glass plates 20 cm × 10 cm (Merck, Darmstadt, Germany) with glass-backed layers silica gel 60 (2 μm thickness). Before use, plates were prewashed with methanol and dried for 3 min at 100C.

***Development and Derivatization.*** The dried extracts of the analyzed samples were weighted and dissolved in methanol (30 mg/mL). Filtered solutions were applied with nitrogen flow. Then the HPTLC plates were developed in the automatic developing chamber ADC 2, saturated with the same mobile phase (Toluene/Ethylacetate 2:8 v/v) for 20 min at room temperature. The length of the chromatogram run was 70 mm from the point of application. The developed layers were allowed to dry on plate heater for 5 min at 120C and then derivatized with a selected solution, including anisaldehyde-sulfuric acid (1 mL anisaldehyde, 10 mL sulfuric acid and 20 mL acetic acid in 170 mL methanol). Finally, the plates were warmed for 5 min at 120C before inspection. All treated plates were then inspected under UV light at 254 or 366 nm or under reflectance and transmission white light (WRT), respectively, at a CAMAG TLC visualizer, before and after derivatization.

**Validation.** Sample solutions of the extracts were found to be stable at 4C for at least 1 month and for at least 3 days on the HPTLC plates. Repeatability was determined by running a minimum of three analyses. RF values for main selected compounds varied ±0.02%. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison. On the contrary, the results were critically dependent on prewashing of HPTLC plates with methanol.

***CC Separations.*** Commercial liquid Raspberry Keton was separated in silica gel column using as mobile phase (Toluene/Ethylacetate 2:8 v/v). Fractions, collected in elution order, were examined in HPTLC, as reported in Fig. 1.

## Spectral Analyses

$^{1}H$ and $^{13}C$ NMR spectra were recorded on Varian (now Agilent Technologies, Santa Clara, CA) Mercury 300 MHz and/or on Bruker Avance III 400 MHz instrument (Bruker, Billerica, MA) using $CD_3OD$ as deuterated solvents; the chemical shift was expressed in ppm from TMS. MS spectra were performed on a Q-TOF MICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source, that operated in the negative and/or positive ion mode. All spectra are obtainable by request.

## HPLC-DAD Analysis

**Extraction Procedure.** After shaking the bottle according to the recommendation reported in the label, 2.5 mL of liquid sample were collected and mixed with 10 mL of ultrapure Milli-Q water into a separating funnel. Then 15 mL of $CH_2Cl_2$ were added to extract the organic phase and the solution was shaken for 2 min. This procedure was repeated four times. The extract was transferred into a 50 mL Falcon that was subjected to centrifugation at 5,000 rpm for 20 min. The supernatant was collected and dehydrated using $Na_2SO_4$. The solvent was removed with a rotary evaporator at $T < 30$C under reduced pressure (60 mbar). Finally, the extract was resuspended in 5 mL of acetonitrile before HPLC analysis.

**Instrument.** The HPLC system consisted of an Agilent Technologies (Palo Alto, CA) HP-1100 series, made of an autosampler and a binary solvent pump, with a diode array detector (DAD). LC separation was performed on a reversed phase column ODS-2 (250 × 4.6 mm i.d., 5 μm) from Merck KGaA (Darmstadt, Germany) thermostatted at 40C,

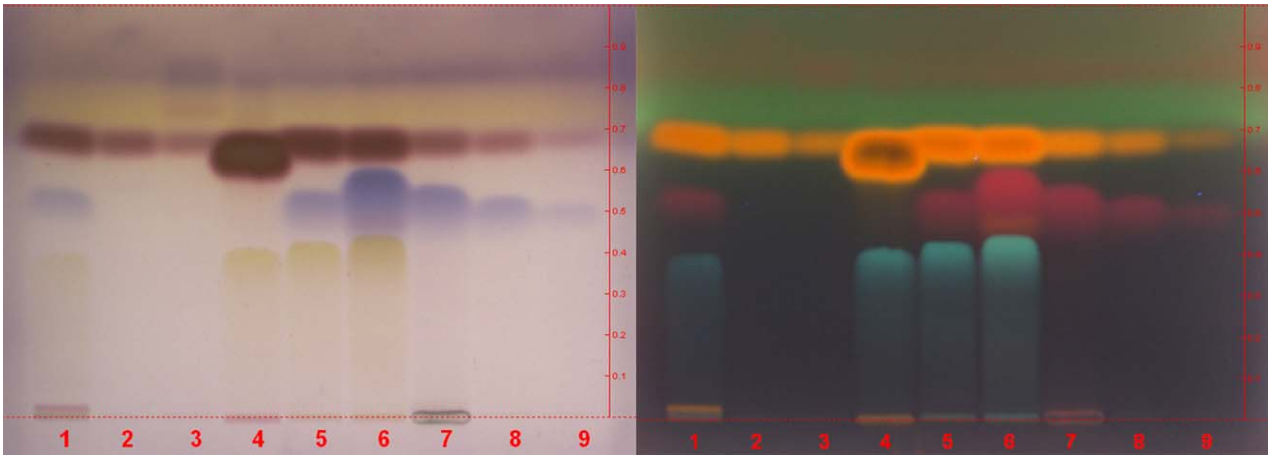


**FIG. 3.** HPTLC ANALYSIS OF COMMERCIAL LIQUID RASPBERRY KETON PRODUCT AND FRACTIONS OF ITS SEPARATION BY COLUMN CHROMATOGRAPHY
Mobile phase: toluene: ethyl acetate 2:8 v/v. Derivatization: anisaldehyde. Revelation: (A) upper and lower white lamp. (B) 366 nm lamp. Tracks: 1, Liquid Rasberry Keton; 2, RK standard; 3–9, column chromatography fractions in elution order.

using mobile phase A (water) and mobile phase B (acetonitrile) in a gradient program with a flow of 1.0 mL/min: 0–15 min: 70% A; 15–35 min: 20% A, 35–40 min: 100% B. The volume of a single injection was 1 µL. After each injection, column was equilibrated for 15 min. For quantitative analysis, the wavelength of 279 nm was used. Quantitative data were collected using Chemstation for LC 3D systems software rev. B.01.03[204] from Agilent (Santa Clara, CA).

**Method Validation.** A 5-point calibration curve was constructed by diluting various amounts of RK (0.1, 0.5, 1.0, 1.5 and 2.0 mg/L) in acetonitrile ($n = 3$ each). To evaluate whether the extraction procedure was reliable for quantifying RK in food supplement, known amounts of the RK standard were added at two fortification levels (1.6 and 4 mg/mL), and the recovery was calculated. The precision of the method was obtained by evaluating the RSD% values for intra- and inter-day measurements ($n = 3$ each).

## RESULTS AND DISCUSSION

The HPTLC analysis evidenced a composition less complex than expected in a botanical. The track 1, reported in Fig. 3, evidenced the presence of a spot at RK's Rf (track 2 at ca. 0.82), as well as few fluorescent spots, one quite near to RK. In order to solve any problem, the product was separated by column chromatography, obtaining a fraction (track 3 in Fig. 3) containing pure RK, as determined by $^{1}H$ and $^{13}C$ NMR and MS data. A second compound present ($Rf =$ ca. 0.53) was assigned to potassium sorbate, a constituent reported in the label, probably added as preservative. Considering the standard concentration used (1 mg/mL), the comparison between tracks 1 and 2 evidences a higher concentration in sample 1.

In the present work we developed an HPLC-DAD method to quantitatively determinate the amount of RK in food supplements (Fig. 4). A 5-point calibration curve was constructed by analysing various amounts (0.1, 0.5, 1.0, 1.5 and 2.0 mg/L) of RK diluted in acetonitrile. The curve showed a good linearity in the whole range of the tested concentrations, with a regression coefficient ($R^2$) of 0.9999. The limits of detection (LOD) and quantification (LOQ), under the present chromatographic conditions, were determined at a signal-to-noise ratio (S/N) of about 3 and 10; they were 0.6 and 2.1 mg/L, respectively. The precision of the method was demonstrated as the intra- and inter-assay RSD% values ($n = 3$) were in all cases below 1.2%. The accuracy was attained as the average recovery obtained by spiking sample at two fortification levels (1.6 and 4 mg/mL, respectively) was 101%. The method developed permitted to quantify in a reproducible and reliable manner the amount of RK in the liquid solution after shaking and centrifugation.

By using the optimized conditions, RK was eluted in less than 8 min from the HPLC system (Fig. 4). The average concentration of RK suspended in the liquid food supplement was calculated as 4.2 mg/mL, that is more than 1,000 times higher than the maximum values reported for raspberry in previous studies (i.e., 20–370, 10–70 and 0.9–17.4 µg/100 g) (Gallois 1982; Borejsza-Wysocki *et al.* 1992; Maquin *et al.* 1981). Therefore, the unusual content of RK in the food supplement may be explained by its addition as a synthetic form. As a matter of fact, synthesis of RK is relative cheap and can be achieved by both chemical and biotechnological processes (Beekwilder *et al.* 2007; Feron *et al.* 2007; Shimoda *et al.* 2007). On the other hand, the high levels of RK detected in

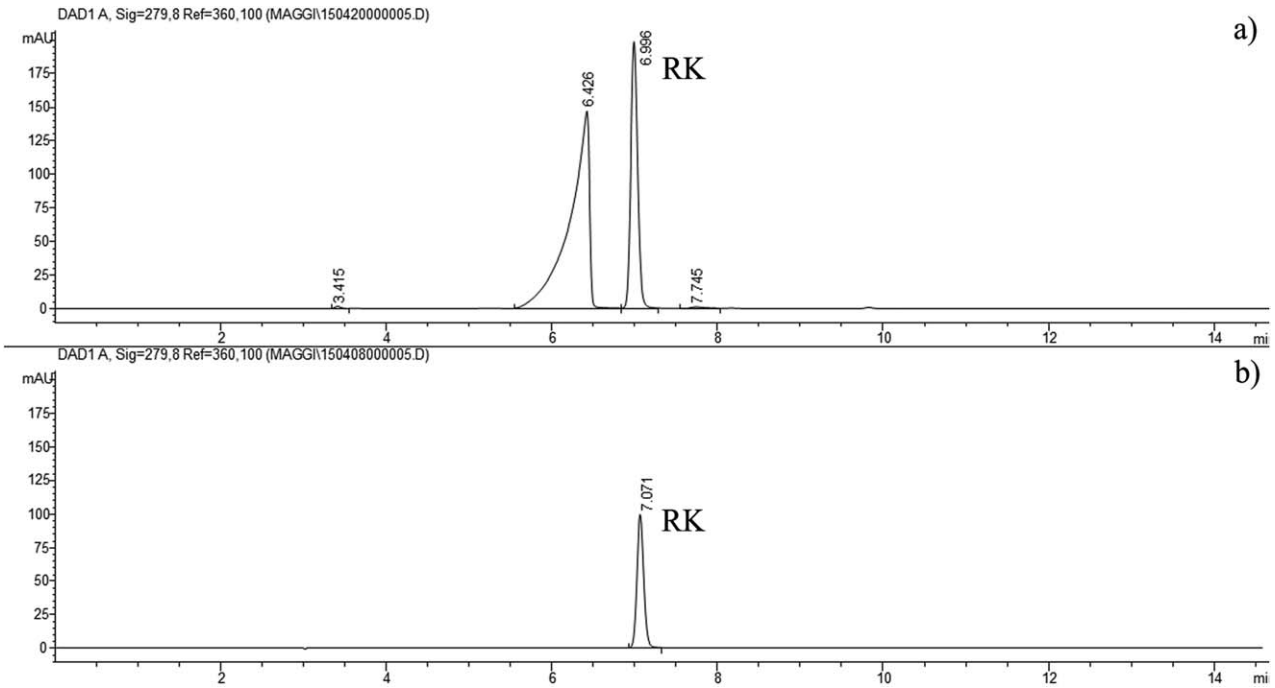


**FIG. 4.** HPLC-DAD CHROMATOGRAM OF EXTRACT OF FOOD SUPPLEMENT (a) AND ANALYTICAL STANDARD OF RASPBERRY KETONE (RK) (b)

the food supplement are not compatible with those derived from its use as a flavouring agent in the European Union (Regulation EC 1334 2008). The recommended doses for RK sold as food supplement on the Internet range from 100 to 1,400 mg/day. The exposure from natural sources only account for a few mg per day, since the RK content in raspberry ranges from 0.0009 to 4.3 mg/kg. This difference arises from exposure of RK as flavouring substance. Therefore, recommended RK daily doses for food supplements are 26–368 times higher than the highest estimated exposure from diet (1.8–3.8 mg/for an adult). This situation is cause of concern and needs to be faced by Food and Health authorities of the EU member states as also reported in a recent toxicological survey (Bredsdorff *et al.* 2015).

## CONCLUSION

The difference between the RK content in the raspberry fruit, as reported in literature, and the analysed marketed product is evident, confirming FSA proposal. Quantity of every single compound in food supplements is considered important for health. As an example, recently EFSA stated the maximum "safe" quantity of caffeine at 400 mg/day. Food supplements are self-assumed, considered safe by the consumers and consumed without any medical control. Possibility of exceeding in quantity is therefore possible. The UK newspaper "Daily Mail," in December 2014 reported that 24-year-old Cara Reynolds died after an overdose of Forza's Rasberry K2 supplement. RK toxicity, as well as effect on body weight, has been recently revised. The margin of safety for RK used as flavouring substance was based on 100 mg/kg bw/day for a person weighing 60 kg. The recent introduction of food supplements containing RK in novel foods in EU, as already regarded by UK, changes the criteria of market authorization. In any case, the data reported in this paper confirm the need of adequate controls on marketed food supplements and the necessity of a complete adherence between labelling and real constitution of the product. As a consequence of this study, the Italian Minister of Health set the maximum levels of RK in food supplements to 50 mg/kg in order to protect consumers from potential risk related to its high intake and to assure its natural origin in the raspberry-based products.

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### REFERENCES

BEEKWILDER, J., VAN DER MEER, I.M., SIBBESEN, O., BROEKGAARDEN, M., QVIST, I., MIKKELSEN, J.D. and HALL, R.D. 2007. Microbial production of natural raspberry ketone. Biotechnol. J. *2*, 1270–1279.

BOREJSZA-WYSOCKI, W., GOERS, S.K., MCARDLE, R.N. and HRAZDINA, G. 1992. (p-Hydroxyphenyl)butan-2-one levels

in raspberries determined by chromatographic and organoleptic methods. J. Agric. Food Chem. *40*, 1176–1177.

BREDSDORFF, L., WEDEBYE, E.B., NIKOLOV, N.G., HALLAS-MØLLER, T. and PILEGAARD, K. 2015. Raspberry ketone in food supplements – High intake, few toxicity – A cause for safety concern? Regul. Toxicol. Pharmacol. *73*, 196–200.

El SOHAIMY, S.A. 2012. Functional foods and nutraceuticals – Modern approach to food sciences. World Appl. Sci. J. *20*, 691–708.

FERON, G., MAUVAIS, G., MARTIN, F., SÉMON, E. and BLIN-PERRIN, C. 2007. Microbial production of 4-hydroxybenzylidene acetone, the direct precursor of raspberry ketone. Lett. Appl. Microbiol. *45*, 29–35.

FLEGAL, K.M., CARROLL, M.D., KIT, B.K. and OGDEN, C.L. 2010. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. J. Am. Med. Assoc. *307*, 491–497.

FOOD STANDARD AGENCY. 2013. Request for information from businesses: Raspberry ketones. Letter, 24 October 2013.

FOOD STANDARD AGENCY. 2014. Raspberry ketones. Letter, 12 March 2014.

GALLO, F.R., MULTARI, G., FEDERICI, E., PALAZZINO, G., NICOLETTI, M. and PETITTO, V. 2012. The modern analytical determination of botanicals and similar novel natural products by the HPTLC fingerprint approach. In *Studies in Natural Products Chemistry* (A.-U. Rahman, eds.) Vol *37*, pp. 217–258, Elsevier, Amsterdam, The Netherlands.

GALLOIS, A. 1982. Rapid determination of *p*-hydroxyphenyl-1-butanone in raspberries by thin layer chromatography. Sci. Aliments *2*, 99–106.

JAMES, P.T., LEACH, R., KALAMURA, E. and SHAYEGHI, M. 2001. The worldwide obesity epidemic. Obes. Res. *9*, 228S–233S.

MAQUIN, F., MEILI, M. and CHAVERON, H. 1981. Determination of the 4-p-hydroxyphenyl)−2-butanone by mass spectrometry. Ann. Falsifications Expertise Toxicol. *74*, 511–521.

NICOLETTI, M. 2011. HPTLC fingerprint: A modern approach for the analytical determination of botanicals. Rev. Bras. Farmacogn. *21*, 818–823.

NICOLETTI, M. 2012. Nutraceuticals and botanicals: Overview and perspectives. Int. J. Food Sci. Nutr. *63*, 2–6.

OGDEN, C.L., CARROLL, M.D., KIT, B.K. and FLEGAL, K.M. 2014. Prevalence of childhood and adult obesity in the United States, 2011-2012. J. Am. Med. Assoc. *311*, 806–814.

REGULATION EC 258. 1997. European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. Off. J. Eur. Commun. *L043*, 1–6.

REGULATION EC 1334. 2008. European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. Off. J. Eur. Commun. *L354*, 34–50.

SHIMODA, K., HARADA, T., HAMADA, H., NAKAJIAMA, N. and HAMADA, H. 2007. Biotransformation of raspberry ketone and zingerone by cultured cells of *Phytolacca americana*. Phytochemistry *68*, 487–492.

TONIOLO, C., NICOLETTI, M., MAGGI, F. and VENDITTI, A. 2013. Determination by HPTLC of chemical composition variability in raw material used in botanicals. Nat. Prod. Res. *28*, 119–126.

WHO/FAO. 2002a. Diet, nutrition and the prevention of chronic diseases. Report of a joint WHO expert consultation. In *WHO Technical Report Series 916*, 28 January to 1 February 2002, Geneva, Switzerland.

WHO/FAO. 2002b. Diet, nutrition and the prevention of chronic diseases. Special issue – Scientific background papers of the joint WHO/FAO expert consultation, 28 January to 1 February 2002. Public Health Nutrition, Vol 7, No. 1(A), Supplement 1001, February 2004, Geneva, Switzerland.

WHO. 2008. Expert Consultation on waist circumference and waist-hip ratio 8 to 11 December 2008, Geneva, Switzerland.