Chlorine flavour perception and neutralization in drinking water

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General introduction
General Introduction

In developed countries, opening water tap is a simple and daily gesture. The water supply does not constitute a problem in everyday life and water consumption is safe. However, it was not always the case in the past. In the 19th century, waterborne infectious diseases remained frequent. The use of chlorine for water disinfection started in England by the end of the 19th century after cholera and typhoid outbreaks. After demonstration of its efficiency, chlorination as disinfection process rapidly spread all over the world and permitted the control of disease-causing organisms. It constituted an essential step in ensuring water safety.

Nowadays, safety does not constitute a concern anymore and consumer’s standards have changed. The taste of tap water became an important concern for consumers. They often report unpleasant taste and especially chlorine taste which is responsible for an important part of their complaints.

Some of the consumers may prefer the use of bottled water as drinking water, even if bottled water consumption would be associated with a higher economic and ecological cost (packaging, transportation). For consumers who resort to alternatives, tap water has a bad image. This explains why it also became an important concern for tap water suppliers. Obviously, a solution to overcome chlorine taste of tap water would be to use chlorination alternatives such as ozonation or UV treatments. However, only chlorine-based disinfectants have residual properties. This property is of high interest since it allows a residual chlorine level being always present into the pipes and preventing micro-organism regrowth during water flow. This especially ensures microbiological quality of water from treatment plant to consumer’s tap.

As a consequence, due to its residual properties but also to its low cost, chlorine is difficult to replace. A way to overcome chlorine taste problem in water would be to reduce its perception without reducing its actual concentration in water. Following this general idea, we explored, in the present Ph.D thesis, several ways to reduce chlorine flavour perception in water.

To address the question of water taste and chlorine taste, Lyonnaise-des-Eaux launched, in 2006, a project entitled “Eau bonne à boire”, in 2006. This project was developed in partnership with Vitagora® which is the Taste-Nutrition-Health Competitive Cluster. The project has for objective to improve the taste of tap water in order to better satisfy French consumers. This project was divided into two parts. The first one relies on the mapping of consumer’s preferences for intrinsic taste of water. E. Teillet was in charge of this component of the project and worked on this topic in the framework of his Ph.D conducted at the CESG (Centre Européen des Sciences du Goût). The second component of the project relies on the investigation of Chlorine taste perception and neutralization. This is the work presented in this thesis manuscript. It was done at the INRA UMR FLAVIC (FLAveur, VIsion & Comportement du consommateur) in collaboration with the CESG, the CIRSEE (Centre
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International de Recherche Sur l’Eau et l’Environnement) which is SUEZ ENVIRONNEMENT’s international expertise center on water and the environment and finally, with Robertet, a company producing and distributing flavours and fragrances.

Before to develop the aims of the Ph.D work, it is important to give some general informations about perception. As aforementioned, some consumers complain about the unpleasant “chlorine taste” elicited during tap water consumption. From a scientific point of view, this “chlorine taste” perception experienced by consumer during water consumption does not rely on taste only, that is the interaction between tastants (chemicals) and the gustatory sensory system. The “chlorine taste” perception should more likely rely on a global percept called Flavour. Indeed, as defined by Beidler (1958, cited in Delwiche 2004) the term flavour rely on “…the sensation realized when a food or beverage is placed into the oral cavity. It is primarily dependent upon the reactions of the taste and olfactory receptors to the chemical stimulus. However, some flavours also involve tactile, temperature, and pain receptors.” The 3 last sensations mentioned are conveyed by trigeminal nerve. Chemicals can also activate this nerve. More recently a review by Delwiche (2004) has highlighted that the different sensory modalities interact, especially within the chemical senses. Multi-sensory interactions especially take place during food and beverages consumption and lead to the formation of a single integrated percept, Flavour, which cannot be decomposed by simple introspection. Thus, during tap water consumption, one can experienced tactile and thermal sensation elicited by water flow in the mouth. Mineral presents in water could activate gustatory receptors located on the tongue. In the same way, chlorine could activate gustatory or olfactory receptors but also the chemosensory receptors located on the trigeminal nerve. Nevertheless, the sensory mechanism involved in its perception remained unknown. In such context, our first aim was to determine the sensory modalities involved in chlorine flavour perception and to investigate the putative link between tap water rejection consumption and sensitivity to chlorine. Chapter 1 contains a short bibliographical review on chlorine flavour perception and its potential link with tap water consumption. This is followed by two publications on this topic and a short summary of the results.

On the basis of the results obtained in the first part of the Ph.D work, we then investigated the sensory interactions that could be associated with chlorine perception which could lead to a reduction of its perception. These aspects of the Ph.D work are presented in two chapters, each devoted to one specific approach. The first approach consisted in the determination of the potential masking effect of the different minerals presents in water. Indeed, as everyone could have experienced, water taste but also chlorine flavour intensity vary from one region to another. The explanation could lie on sensory interaction between perception elicited by the ions contained in water and chlorine flavour perception. We determined the ions varying in tap water distributed in France, their impact on water taste and finally their impact on
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chlorine flavour perception. This approach is developed in Chapter 2 which contains a short bibliographical review on water components that may elicit a sensory perception and their potential perceptive interaction with chlorine. After this bibliographical review, 4 publications are included and describe the experiments undertaken on this topic. A short summary of the results could be found at the end of the chapter.

The second approach followed in this part of the Ph.D work aimed to determine the potential interactions between selected odours and chlorine flavour. We especially tried to select odours that have the ability to reduce chlorine flavour perception. To avoid any perception of flavours in water that would likely be inadapted to tap water distribution, odorants were considered at sub/perithreshold concentrations. This work was partly performed in collaboration with Robertet S.A. and is presented in Chapter 3. This chapter contains a short bibliographical review on perceptual interactions between odours followed by two publications on this topic, and a short summary of the results.
Chapter 1.

Chlorine flavour perception
1. Introduction

French public health code mentions that waters intended for human consumption have not to contain a number or a concentration of micro-organisms, parasites or other substances constituting a potential danger for the health of persons (de Forges et al. 2009). This type of regulations also exists in the US or in other European countries for example. Some of them defined requirements or guidelines concerning disinfectant residuals within the distribution system (Doré 1989; Connell 1996). For example, French Public Health Authority imposes a minimal value of 0.3 mg/L Cl₂ at the treatment plant outlet and a minimum of 0.1 mg/L at tap (Journal Officiel 2001). Thus, chlorine addition is one of the most common treatments used to ensure tap water bacteriological quality. Chlorination had spread out all over the world due to its low cost and its efficiency. Indeed, it inactivates various types of micro-organisms such as Cryptosporidium, Gardia, but also various types of bacteria and viruses (Connell 1996; Haas 1999; Haas and Engelbrecht 1980). Chlorination is usually ensured by gaseous chlorine (Cl₂) inlet in water. Chlorine reacts with water to form hypochlorous acid (HOCl/ClO⁻). pKa of this acid has a value of 7.5.

![Figure 1: Evolution of the proportion of the different forms of chlorine according to pH](image)

Since the pH value for water is usually comprised between 6 and 8, chlorine is mainly present in its associated form (HOCl) and dissociated form (ClO⁻) into the pipes (Doré 1989). It has been demonstrated that disinfection efficiency is higher when pH is lower, that is to say when associated form is present in greater quantity. One possible explanation lies on the negative charges coated on the surface of bacterial membranes. Thus, the associated form (HOCl) can penetrate more easily into the bacteria than the dissociated form (ClO⁻). Thereby,
it alters enzymes and upsets internal machinery due to oxidation reactions (Connell 1996). Due to its high reactivity, chlorine not only reacts with micro-organisms, but also reacts with chemicals present in water within the distribution system. These chemical reactions often lead to by-products formation and off-flavours development (Froese et al. 1999; Heim and M. 2007). Specific tools such as odour wheel are available for targeting off-flavours in drinking water (Suffet and Rosenfeld 2007) and identify locations vulnerable to taste and odour problems (Proulx et al. 2007). However, chlorine by itself elicits a flavour which constitutes one of the major complaints advocated by consumers. This has been often noticed during various surveys all over the world (Chotard 2008; Miquel 2003; Suffet et al. 1996). Thus, 35% of French consumers who participated in the SOFRES/C.I.Eau 2008 annual survey complained about water bad taste quality and especially the bad taste conferred to tap water by chlorine (Chotard 2008).

As far as chlorine flavour is concerned, several scientific publications investigated the link between tap water consumption, taste of water and risk perception associated with tap water consumption (Anadu and Harding 2000; Jardine et al. 1999; Levallois et al. 1999). Obviously, other factors are susceptible to affect water consumption behaviour such as cost of water or quality of raw water. Other publications were interested in chlorine flavour thresholds measurements. Bryan et al. (1973) were the first to measure chlorine thresholds. Theses authors measured flavour threshold of halogens and demonstrated that the chlorine flavour detection threshold decreases according to pH. Thus, threshold increases as pH and the hypochlorous acid dissociated form (ClO-, non-volatile) increases. On the opposite, an increase of the volatile associated form (HOCl) is associated with a threshold decrease. These observations suggest that it is the volatile associated form of chlorine (HOCl) which elicits chlorine sensory perception. However, chlorine detection mechanisms as well as sensory modalities involved in chlorine flavour perception remain unknown.

Chlorine perception threshold have been measured in various conditions. Krasner and Barrett (1984) determined that thresholds were 0.28 mg/L Cl₂ for odour and 0.24 mg/L Cl₂ for flavour. This means that olfaction at least is involved in chlorine flavour detection. Then, Piriou et al. (2004) compared chlorine flavour thresholds for different type subjects. These authors found a significant difference between a group of trained subjects (0.05 mg/L Cl₂) and a group of untrained subjects (0.2 mg/L Cl₂). They also compared the threshold in the US (1.1 mg/L Cl₂) and in France (0.2 mg/L Cl₂) and found a significant difference. The same year, Mackey et al. (2004a) measured chlorine flavour threshold in the U.S. and failed in finding a significant link between tap water consumption and chlorine flavour sensitivity. However, it is noteworthy that only a few publications investigated the link between sensitivity to chlorine and water appraisal. This was done in Canada, by Turgeon et al. (2004). These authors demonstrated that consumers supplied with tap water containing a residual chlorine...
level greater than the threshold measured by Krasner and Barrett (1984) were less satisfied by tap water quality and perceived more risks. Data published up today did not clearly evidence a link between tap water consumption and chlorine flavour perception. Therefore one important question remains to be answered: is chlorine flavour a determinant of tap water rejection?

In order to try to answer this question, we decided, to fully investigate the link between tap water consumption and chlorine flavour perception. Since chlorine perception mechanisms are not clearly elucidated, our strategy was not limited to the measurements of flavour thresholds. We also integrated the measurement of other hedonic and cognitive dimensions. To do so, we compared the chlorine perception of two groups of consumers: a group including exclusive tap water consumers and a group including exclusive bottled water consumers. Sensory measurements performed with these two groups had for aim to compare their sensitivity but also their perception of chlorine through measurements including intensity, preference and acceptability. This work is detailed in a first paper (publication 1). In a second publication, we present a series of data obtained with the same consumers. This part of the work aims to determine the sensory modalities involved in chlorine flavour perception and their respective activation threshold values.
Chapter I – Chlorine flavour perception – Publication 1

2. Publication 1:

Tap water consumers differ from non-consumers in chlorine flavour acceptability but not sensitivity.

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1. Introduction

Adding chlorine to tap water is one of the most common treatments to ensure its bacteriological quality. Used for the first time in England in the 1880s, chlorine treatment of drinking water has spread all over the world. Chlorine inactivates various types of microorganisms and its residual properties help to prevent micro-organism regrowth during water flow in the pipes (Connell 1996; Al-Jasser 2007). Indirect sensory effects of water chlorination, linked to the development of off-flavours due to by-products, have been studied (Froese et al. 1999; Heim and M. 2007). Methodological developments were proposed to identify locations vulnerable to taste and odour problems (Proulx et al. 2007) and specific tools (e.g., the odour wheel) are now available for targeting off-flavours in drinking water (Suffet and Rosenfeld 2007; Burlingame et al. 2007; Suffet et al. 1988). Beyond off-flavours development due to chlorination by-products, chlorine flavour by itself constitutes one of the major complaints against tap water. In 1996, chlorine taste was the third most reported taste default of tap water in the US (Suffet et al. 1996). In France, an annual survey performed in 2008 by SOFRES/C.I.EAU (Chotard 2008) indicated that 40% of the interviewed consumers reported an unpleasant water taste and 34% an unpleasant chlorine taste. Due to the unpleasant taste of tap water, consumers may prefer bottled water as drinking water, even if bottled drinking water consumption would be associated with a higher economic and ecological cost (Milmo 2006). The 2008 SOFRES/C.I.EAU survey showed that 41% of consumers mainly drink bottled water and 26% are exclusive consumers of bottled water (Chotard 2008). In Canada, Levallois et al. (1999) also found that bottled water consumption
was mainly due to organoleptic reasons. Turgeon et al. (2004) showed that the perception of tap water quality is closely related to the residual chlorine level: people living near a treatment plant who may receive a higher chlorine level in their tap water were generally less satisfied by tap water quality and perceived more risks associated with it than people living far from the plant. It was reported that, in the US, bottled water drinkers have three main categories for decisions: safety of water; healthfulness of the water; and taste of the water (Mackey et al. 2004a,b). Consumers supplied with tap water containing a residual chlorine level greater than 0.24 mg/L Cl₂ were less satisfied with tap water when compared to consumers receiving lower concentrations (Turgeon et al. 2004). This chlorine level likely corresponded to a chlorine detection threshold, as measured by Krasner and Barrett (1984). When taken together, these studies underline the role of chlorine flavour in the lack of tap-water acceptance by consumers. Piriou et al. (2004) showed that chlorine detection thresholds in water vary according to subject experience, the threshold for experienced subjects being lower. In the same way, French consumers’ thresholds were lower than American ones, revealing cultural differences. Flavour is defined as a sensory percept induced by food or beverage tasting. It relies mainly on the functional integration of information transmitted by the chemical senses: olfaction, gustation, oral and nasal somatosensory inputs (Thomas-Danguin 2009). Once in the mouth, volatile compounds are retronasally conveyed to the nasal cavity where they are inclined to activate the olfactory receptors located on the top of the olfactory cleft and the trigeminal fibers inserted into the whole nasal mucosa. Soluble compounds could be dissolved into the saliva and some of them could further be detected by the gustatory cells of the taste buds and the trigeminal fibers also inserted in the oral mucosa (Laing and Jinks 1996; AFNOR 1992). These three sensory modalities are simultaneously activated and interact to create an integrated unique perception, flavour, which cannot be decomposed by simple introspection. As far as chlorine flavour is concerned, the mechanisms of perception remain poorly investigated. If chlorine flavour detection threshold in water has been evaluated several times, it is not however known whether this threshold, which reflects a subject’s (or a group of subjects’) mean sensitivity to chlorine, is different for consumers who regularly use tap water as drinking water as compared to consumers who prefer bottled water. In summary, the bibliographical review showed that, on the one hand, chlorine flavour constitutes one of the major complaints against tap water. On the other hand, through available consumers inquiries performed with questionnaires, it appeared that several consumers choose tap water alternatives and drink bottled water for taste reasons. Taking into account these results, we set the hypothesis that the perception of chlorine flavour could be a reason why a proportion of consumers prefers bottled water to tap water. Additionally, we hypothesized that consumers who choose tap water alternatives may be more sensitive to chlorine flavour as
compared to consumers who accept tap water as drinking water. Therefore, we set out to determine whether chlorine flavour sensitivity could be a driver for tap water acceptability. To do so, we compared the sensitivity to chlorine flavour of a group of consumers who usually drink tap water and a group of consumers who never drink tap water. Since several forms of chlorine are used in water, and pH affects the speciation of chlorine (Bryan et al., 1973), chlorine flavour was generated from free available chlorine in Evian water at a fixed pH value. We conducted two experiments in order to determine whether tap water consumers differ from non-consumers both in chlorine sensitivity and acceptability.

The first experiment was dedicated to measure chlorine flavour detection threshold for a group of tap water consumers and a group of non-consumers. To do so, we used the constant stimuli procedure which has been recommended for its fine resolution (Wise et al. 2008). This method has the advantage of threshold estimation based on psychometric function modeling and is especially recommended for individual threshold estimations. The aim of the second experiment was to investigate putative differences between the two groups in chlorine suprathreshold sensitivity and liking. Additionally, acceptability of chlorinated water as drinking water was measured for both groups using water solutions including suprathreshold chlorine concentrations.

2. Materials and methods

2.1. Stimuli

Chlorinated water samples were obtained by adding sodium hypochlorite (NaOCl~15%, RECTAPUR, VWR international, France) to Evian water (La Bourgogne, Dijon, France). Evian water was chosen because of its neutral taste due to medium mineral content (Teillet et al. 2008) and its compositional stability. Evian water had been also chosen in previous studies (Piriou et al., 2004; Mackey et al., 2004a,b). Evian water was purchased in 1 L glass bottles from the same lot. In the first experiment, the concentrations of chlorine (mg/L Cl₂) in the samples were the following: 0.01 mg/L, 0.03 mg/L, 0.06 mg/L, 0.1 mg/L, 0.17 mg/L, 0.32 mg/L, 1 mg/L, and 3 mg/L. In the second experiment, the concentrations of chlorine (mg/L Cl₂) in the samples were the following: 0 mg/L, 0.03 mg/L, 0.1 mg/L, 0.3 mg/L, 1 mg/L, 3 mg/L, and 10 mg/L. Since sodium hypochlorite solutions provide free chlorine but also sodium ions which contribute to taste, control solutions used in discrimination tests needed to be compensated for sodium (Lugaz et al. 2002). Control solutions were prepared adding sodium chloride (NaCl, Jera, France) to reach the same sodium content as the eight chlorine solutions used in experiment 1, respectively: 0.02 mg/L, 0.05 mg/L, 0.1 mg/L, 0.17 mg/L, 0.28 mg/L, 1.65 mg/L, and 4.95 mg/L. Because of chlorine’s high volatility and degradation by sunlight (UV), chlorinated solutions were prepared daily and stored until tasting, i.e., for a
maximum of eight hours, in brown glass 500 mL flasks equipped with brown glass stoppers. Before use, flasks and stoppers were heated at 400°C for 2 h and then 3 times rinsed with a sodium hypochlorite solution (1%) in order to minimize chlorine demand due to residual compounds which could remain on the glass. As a consequence, the expected chlorine content of the delivered samples was controlled by free and total chorine measurement. In the absence of chlorine demand, these parameters are assumed to be close to the expected value. Both parameters were controlled daily in each flask just after solution preparation and at the end of each sensory session. These controls were performed through a procedure (pocket colorimeter II, Hach Lange) adapted from the DPD protocol for spectrophotometry (APHA-AWWA-WEF 1998). Temperature and pH of each solution were controlled (Solitrode Pt 1000 and 781 pH/Ion meter, Metrohm, Courtaboeuf, France). For sensory tasting, samples of 10 mL were delivered in plastic glasses. The absence of off-flavour due to Evian water and the cups was checked. Additionally, the absence of chemical reaction between chlorine and the plastic material was checked through free and total chlorine measurements.

2.2. Consumers

Two hundreds of consumers randomly selected from the Dijon area were first contacted for an inquiry on their water consumption and general food habits. On the basis of their responses, 72 out 200 were invited to participate to sensory deficiency screening tests and to a more extensive questionnaire on their drinking water habits. Screening tests consisted in the European test of olfactory capabilities (ETOC, Thomas-Danguin et al. 2003), a test to evaluate subject’s ability to rank six chlorine solutions with increasing sodium hypochlorite concentrations and a mental concentration test (Bourdon Test, Lesschaeve 1997). This selection was done in order to avoid highly sensory deprived subjects and to be sure to include exclusive tap water consumers or exclusive tap water non-consumers in the two dedicated groups. In the end 40 out of 72 consumers participated to the study. Only one consumer was excluded because of his results to the screening tests (low score at ETOC). The other non-selected consumers could not participate because of their non-availability during the whole testing sessions, or because of their water consumption which did not fit with the strict selection criteria. On the one hand, consumers included in the tap water consumer group were people who daily drink chlorinated tap water without using any devices or processes to reduce chlorine flavour. Following the French standard concentration, these consumers receive, at tap, a chlorine concentration between 0.1 and 0.3 mg/L. Within the Dijon area consumers receive water with a medium TDS value (305 mg/L). On the other hand, consumers included in the tap water non-consumer group were people who do not drink tap water and declared themselves to be exclusive bottled water consumers.
The participants signed an informed consent form but the aim of the experiment was not revealed. They were asked not to modify their water consumption during the study and to avoid smoking, drinking and eating at least one hour before each session and to avoid using perfume the day of the test. Subjects were paid for their participation. Twenty tap water consumers (9 women, 11 men) with a mean age of 38±11 years and 20 tap water non-consumers (14 women, 6 men) with a mean age of 43±14 years participated in the first experiment. Only 35 of the previous 40 consumers took part in the second experiment: 18 tap water consumers (8 women, 10 men) with a mean age of 38±11 years and 17 non-consumers (11 women, 6 men) with a mean age of 41±14 years.

2.3. Experimental procedure

All experiments were performed in a room dedicated to sensory analysis following HACCP and Research Quality Insurance Standards. Consumers were placed in separate booths and their responses were collected using software dedicated to sensory analysis (FIZZ, Biosystèmes, Couternon, France). Consumers received 10 mL stimulus samples in plastic glasses coded with a three-digit number.

2.3.1. Experiment 1: chlorine flavour threshold measurement

Flavour thresholds were measured according to the constant stimuli procedure (AFNOR 2002). This procedure allows psychometric function modeling on the basis of detection probability measurement for increasing stimulus concentrations. In our experiment, the detection probability was measured using a three-alternative forced choice (3-AFC) discriminative test. In this test, three samples were simultaneously delivered to a consumer. One of the samples contained a chlorinated stimulus, at one of the eight chlorine concentrations, and the two other samples contained the corresponding control solution (balanced for sodium content).

Subjects had to taste the three samples and to decide which one contained the chlorinated stimulus. Four repetitions were performed by each consumer at each concentration levels in order to ensure a sufficient statistical power (Schlisch 1993).

A total of 32 tests (3-AFC) were performed by each subject during two one-hour sessions (16 tests in a session; the two sessions spaced by a week). The sample presentation order was the same for all consumers; however, the location of the odd sample in one 3-AFC test was random for each trial. Subjects had to wait at least 140 s between each trial. They were instructed to rinse their mouth with Evian water during this inter-trial interval.
2.3.2. Experiment 2: chlorine flavour intensity, liking and acceptability rating

Chlorine flavour intensity, liking and acceptability were evaluated within a third session (lasting 1.5 h). The session was divided into two blocks; the first one was dedicated to liking and acceptability ratings and the second to intensity rating.

The sample presentation order was randomized and different in each block. Four different presentation orders were used within a block. Within the first block, liking for each sample was rated on a 23 cm linear scale from “I don’t like this sample” to “I like this sample” (Fig. 1). Ratings on this scale were normalized to obtain a score between 0 and 10. For acceptability, consumers were asked “If this water was daily delivered to your tap, would you drink it?” They were instructed to answer by yes or no (Fig. 1). Within the second block, consumers rated chlorine flavour intensity on a 23 cm linear scale from “It doesn’t taste chlorine” to “It strongly tastes chlorine” (Fig. 1). Ratings on this scale were normalized to obtain a score between 0 and 10. Subjects had to wait at least 140 s between each sample.

They were instructed to rinse their mouth with Evian water during this inter-trial interval.

(1)

(2)

(3)

Fig. 1 – Scales and question used to measure liking (1) acceptance (2) and intensity (3).

2.4. Data analysis

All statistical analyses were performed using SAS release 9.1.3 (SAS Institute Inc., Cary, NC). Sensory data analyses were based on mixed modelling procedures which allow considering subjects as a random factor. This means that it could be considered that the consumers included in the tap water consumer group (respectively the non-consumer group) were randomly selected within the tap water consumer population (respectively the non-consumer population) and could be considered as representative of the French tap water consumer population (respectively the non-consumer population; Pinheiro and Bates 2000).
2.4.1. Experiment 1

In the 3-AFC tests, correct answers were coded with a 1 and incorrect with a 0. Each subject performed 4 repetitions (n = 4) at each concentration. The number of correct answers Y is distributed as a binomial law \([B(n,p)]\) where the correct answer probability \(p\) combines correct answers occurring by chance (i.e., 1/3) and correct answers due to real detection (\(p_d\), equation (1)).

\[
(1) \quad p = p_d \cdot \frac{1}{3} (1 - p_d)
\]

\(p_d\), the detection probability, follows a sigmoid function of the logarithm of the stimulus concentration (psychometric function). Therefore, the logit function of \(p_d\) is assumed to be linearly linked to the logarithm of the stimulus concentration (equation (2)).

\[
(2) \quad \text{Logit}(p_d) = \ln\left(\frac{p_d}{1 - p_d}\right) = \alpha + \beta \log_{10}(\text{concentration})
\]

In equation (2), the slope \(\beta\) is assumed to be constant whereas the intercept varies according to the subject sensitivity. Indeed, since each subject’s sensitivity to the stimulus is different, the constant term \(\alpha\) was supposed to be random. Therefore, we introduced for each subject a constant term \(\alpha_s\) randomly distributed according to the normal law around the average value \(\alpha\) (equation (3)).

\[
(3) \quad y \sim B(n,p) \quad \text{with p such as:}
\]

\[
\log\left(\frac{p - \frac{1}{3}}{1 - p}\right) = \alpha_s + \beta \log_{10}(\text{concentration}) \quad \text{and}
\]

\[
\alpha_s \sim N(\alpha, \sigma_s^2)
\]

By definition, the threshold is the concentration detected with a probability equal to 50%, that is, \(p_d = 0.5\) (AFNOR 2002). Using equation (3), the threshold could be estimated for each group of consumers (equation (4)) but also for each subject (equation (5)) even if some of them did not detect 100% of the trials at the highest concentration level.

\[
(4) \quad \log_{10}(\text{Threshold}) = -\frac{\alpha}{\beta}
\]

\[
(5) \quad \log_{10}(\text{Threshold}) = -\frac{\alpha_s}{\beta}
\]

The modeling was performed using SAS and the parameters were estimated using the NLMIXED procedure. A first model (model 1) was used to estimate the mean threshold for the whole set of consumers. Additionally, and since we hypothesized that the two consumer groups might differ in their chlorine flavour thresholds, a second model (model 2) was also
implemented to take into account this putative sensitivity difference. In this model, the $\alpha$ term is assumed to be different between the two groups and could have a different value ($\alpha_{\text{TWC}}$ for tap water consumers and or $\alpha_{\text{TWNC}}$ for non-consumers). The second model differs from the first one only in the $\alpha_s$ term and can be written $\alpha_s$ follows:

$$\alpha_s \sim N(\alpha_{\text{TWC}} + \Delta\alpha, \sigma_s^2)$$

Thus, $\alpha_{\text{TWC}}$ is used to take into account the sensitivity of tap water consumers and $\Delta\alpha$ to account for a putative sensitivity difference between both the consumer groups. As a consequence, $\alpha_{\text{TWNC}}$ is the sum of $\alpha_{\text{TWC}}$ and $\Delta\alpha$.

Therefore, testing the difference between both the groups means testing the significance of $\Delta\alpha$. This can be achieved by comparison of the two models (model 1 and model 2), through a nested model comparison using likelihood ratio tests. This comparison aimed to determine whether model 2 better fits the data than model 1. In other words, this comparison allowed us to determine whether the sensory difference between tap water consumers and non-consumers is statistically significant.

### 2.4.2. Experiment 2

Intensity and liking rating data were submitted to an ANCOVA (Analysis of Covariance) with subjects as random factor and chlorine concentration as covariate (GLM procedure of SAS). Post hoc comparisons of means were performed with a Bonferroni adjustment for multiple comparisons. Acceptability data (yes or no answers) were analyzed through the Generalized Equation Estimation for binary data (GENMOD procedure of SAS) with subjects as a repeated effect (Zeger et al. 1988).

### 3. Results

Free and total chlorine contents of the samples delivered to the panelists were controlled and physico-chemical data confirmed that, for both experiments, each solution contained the expected amount of free and total chlorine (Table 1). Multiple comparisons of means revealed that chlorine concentration differed significantly between each level with the exception of the two first levels (Table 1). Solutions had a mean pH value of 7.54±0.01 and the mean temperature of the samples was of 21.1±0.1°C.
3.1. Experiment 1: chlorine flavour threshold measurement

The objective of this experiment was to determine the chlorine flavour detection threshold for a group of tap water consumers and for a group of non-consumers. We especially wanted to determine whether these two groups had a different mean sensitivity toward chlorine flavour. Individual psychometric functions were recorded for each panelist. An example of this function is presented in Fig. 2.

Data analysis recommended by the International Organization for Standardization for sensitivity thresholds measurement (AFNOR 2002) is based on a modeling of individual psychometric functions leading to individual threshold estimation. This was done following a global modeling approach in which (i) the number of correct answers is assumed to follow a binomial law, (ii) the logit of the probability of detection is assumed to be linearly related to the logarithm of the concentration, and (iii) each subject’s sensitivity to the stimulus is
assumed to be randomly distributed, according to a normal distribution. Two models were actually compared: the first one was used to estimate the mean threshold for the whole set of consumers whereas the second one took into account a putative sensitivity difference between the two groups of panelists. The estimated thresholds for the tap water consumer group were 0.21 mg/L (SEM = 0.16) and 0.09 mg/L for the non-consumer group (SEM = 0.07). However, the comparison (nested model likelihood ratio test) revealed no significant difference ($\chi^2 (1, N=40) = 0.6, p = 0.44$) between the two models, which demonstrated that the data were equally well-described by both models. As a consequence, chlorine detection threshold had to be estimated using the first model which implies that the difference between detection thresholds of both groups should not be considered to be significantly different.

Therefore, the mean chlorine flavour detection threshold estimated through the first model was 0.14 mg/L (SEM = 0.08) for the whole set of consumers. Individual thresholds were also estimated. Their distribution indicated that 65% of the consumers had a chlorine detection threshold lower than 0.3 mg/L Cl$_2$ (Fig. 3) which corresponds to the French standard concentration to be fixed at the exit of the treatment plant. It is noteworthy that the dispersion of chlorine flavour detection thresholds was found to be large.
3.2. Experiment 2: chlorine flavour intensity, liking and acceptability rating

The second experiment was dedicated to supra-threshold chlorinated water flavour intensity, liking and acceptability assessments. Data were especially analyzed to test differences between tap water consumers and non-consumers. A two-way ANCOVA (subject within group, group and chlorine concentration as covariate) was performed on both flavour intensity and liking and revealed a significant effect of the chlorine concentration on both intensity ($F_{(1,204)} = 77; p<0.0001$) and liking ($F_{(1,209)} = 38; p<0.0001$). Fig. 4 shows that intensity ratings increased with concentration. Surprisingly, the stimulus–response curve did not follow the theoretical stimulus–response function (Chastrette et al. 1998). Intensity increased from 0 to 0.3 mg/L Cl$_2$, then, reached a plateau and followed a final upward trend from 1 mg/L Cl$_2$ up to 10 mg/L. As far as hedonic rating is concerned, the liking was found to decrease when the stimulus concentration increased with a plateau between 0.1 and 3 mg/L Cl$_2$. ANCOVA results indicated that tap water consumers were not different from non-consumers as far as chlorine flavour intensity rating was concerned (group: $F_{(1,33)}=1.3; p=0.26$). However, tap water non-consumers expressed a lower liking for chlorinated solutions as compared to regular consumers (group: $F_{(1,33)}= 4.0; p=0.05$) (Fig. 5). Generalized equation estimation for binary data was applied to analyze acceptability data. Consumer group and chlorine concentration were tested as factors. A significant effect of chlorine concentration was found on acceptability ($z_{(1,242)}=3.93; p<0.0001$). Chlorine flavour acceptance decreased with increasing chlorine concentration and followed the same pattern as hedonic ratings (Fig. 6). In addition, a highly significant effect of the group of consumer on acceptability was noticed ($z_{(1,242)}=3.1; p=0.002$). Tap water consumers declared being more inclined to consume the chlorinated water samples delivered in this experiment than tap water non-consumers.
4. Discussion

First of all, our recruitment procedure as well as our data analyses allow to consider participants included in consumer groups as a random factor. Therefore, it could be considered that the consumers included in the tap water consumer group (respectively the non-consumer group) were representative of the exclusive tap water consumer population (respectively the non-consumer population). Consequently, our results should not be restricted to the community of the people tested but could be extended to the two populations of French consumers.

Our results did not provide evidence of any difference in chlorine flavour sensitivity, at the detection threshold level, between tap water consumers and non-consumers. The detection threshold for both groups was 0.14 mg/L which is consistent with previously published thresholds: 0.16 mg/L Cl₂ (Bryan et al., 1973), 0.24 mg/L Cl₂ (Krasner and Barrett, 1984) and 0.2 mg/L Cl₂ (Piriou et al., 2004). We also observed a large inter-individual difference in
chlorine flavour sensitivity. These results are especially in agreement with those obtained by Mackey et al. (2004\textsuperscript{a,b}), who studied public perceptions of chlorine flavour in drinking water and their impact on customer’s choices with respect to drinking water consumption habits. Indeed, these authors did not find a significant difference between tap water consumers and users of tap water alternatives but noticed a large inter-individual difference in sensitivity. We did not find a significant difference between the two groups of consumers for chlorine flavour supra-threshold intensity perception. However, we observed a difference between tap water consumers and non-consumers for liking and acceptability of chlorinated solutions. Beyond inter-individual differences, it is important to notice that intensity relies on different perceptual processes as compared to hedonic perception (Bensafi et al. 2003). Indeed, liking and intensity judgment elicit activation in similar brain areas but pleasantness also elicits activation in the hypothalamus, which is known to be involved in affective processing which requires access to information about internal states (Zatorre et al. 2000). In other words, pleasantness judgments imply a decision: in this case, whether one odour might indicate something good to eat or something that could make one sick.

It is thus not surprising that, for similar flavour intensity levels, tap water non-consumers appreciated chlorinated solutions less than tap water consumers. This was confirmed by our findings, which demonstrated that tap water non-consumers accepted less chlorinated solutions as water to be drunk than tap water consumers. It is likely that acceptability judgments rely on high cognitive aspects, since consumers were asked to choose if they would drink the chlorinated solutions in the context of daily tap water consumption. Whether consumer sensitivity and perception could be a driver for food liking and acceptability remains an unanswered issue. For example, regular caffeine users were found to have higher detection thresholds for caffeine than non-users (Tanimura and Mattes 1993). In contrast, Delahunty and Lee (2007) evidenced that acceptance and fruit consumption were related to sweet and sour liking but not to sensitivity toward these tastes. Beside sensitivity, many factors have to be taken into account to understand food choice. Hudon et al. (1991) who investigated water consumption habits in Canada, through a consumer telephone survey, noticed a group of consumers whose tap water representation was quite positive with no indication of any health risk. These consumers also declared to drink tap water and found it to be of good organoleptic, chemical and bacteriological quality. Conversely, another group of consumers, whose tap water representation was clearly negative on the same items, declared they drank bottled water. This link between tap water representation, safety and organoleptic properties was also suggested by McGuire (1995): consumers who detect off-flavours in their drinking water likely associate these perceptions to a lack of water safety, even if there is no link between organoleptic effects of drinking water contaminants and their actual toxicity (Young et al. 1996). Torobin et al. (1999) also noticed a correlation between
perceived safety and actual water taste and suggested that taste does shape safety perception. Our data showed that increased chlorine flavour perception (intensity) was associated with a more pronounced decrease of water acceptability in tap water non-consumers. As a result, chlorine flavour seems to play in fine against tap water acceptability as a marker of safety representation. It is nevertheless noteworthy that other extrinsic factors such as environmental perception (e.g., raw water bad quality) have also been highlighted to influence consumer choice (Bontemps and Nauges 2006). Our data suggested that most of French consumers perceive chlorine flavour at tap since a decree of the French Public Health Authority imposes a minimal value of 0.3 mg/L Cl\textsubscript{2} at the treatment plant outlet and a minimum of 0.1 mg/L at tap (Ministère de la santé, de la famille et des personnes handicapées, 2001). Indeed our data predicted that 65% of the consumers may have a threshold below 0.3 mg/L Cl\textsubscript{2} (mean threshold value at 0.14 mg/L Cl\textsubscript{2}). Once perceived, chlorine flavour may constitute a marker of water acceptability as a beverage depending on individual tap water representation. Tap water consumption could be seen as the result of a subtle balance between sensitivity, actual chlorine content of tap water and tap water representation. The two last parameters are susceptible to be modified through water supply process improvement or tap water information delivery. Another outcome of the present study is the non-classical stimulus–response function observed for chlorine flavour intensity. Indeed, it is commonly admitted in chemosensory perception that the theoretical stimulus–response function should follow a monotonically increasing sigmoid shape from the threshold plateau to the saturation plateau seldom reached at high stimulus concentrations (Chastrette et al., 1998). However, our data showed that chlorine flavour intensity increased from 0 to 0.3 mg/L then reached a plateau until 1 mg/L and finally followed a final upward trend up to 10 mg/L.

This observation seems to be consistent as the same profiles were observed for liking and acceptability measurements, also showing a plateau in this range of concentrations. The physico-chemical controls performed confirmed that these observations cannot be due to errors in chlorinated solution preparation, neither to temperature differences between samples (Whelton and Dietrich 2004) nor to solution conservation problems. One possible explanation would rather be found in the fact that chlorine flavour is an integrated perception which potentially combines several sensory modalities (Thomas-Danguin 2009; Auvray and Spence 2008; Small and Prescott 2005). Thus, the non-classical shape of the stimulus–response curve could be explained by the implication of at least two sensory modalities in the chlorine flavour perception. A first sensory mechanism would be involved until the first plateau (first saturation mechanism between 0.3 and 1 mg/L) followed by a second sensory mechanism. In previous studies, chlorine flavour perception was described through Flavour Profile Analysis (FPA) method using a rather limited range of concentrations (Krasner and
Barrett, 1984) and suggested that chlorine flavour included an olfactory dimension. However, sensory mechanisms implicated in chlorine perception remained largely unknown. Gustatory and trigeminal systems could likely be involved in chlorine flavour perception. Indeed, most odorants also activate the trigeminal nerve and trigeminal thresholds are usually higher than olfactory ones. Therefore, the second part of the observed stimulus–response curve at concentrations higher than 1 mg/L Cl₂ might be reconcilable with trigeminal system activation. It is noteworthy that following such a hypothesis, these high chlorine concentrations also correspond to an important drop in liking and acceptability which suggests that the second sensory mechanism activated would have a great role in chlorinated tap water lack of acceptability. Nevertheless, this hypothesis remains to be confirmed by further investigations.

5. Conclusion

In the present study, regular tap water consumers were found to be as sensitive to chlorine flavour as bottled water consumers. However, bottled water consumers showed a lower appreciation of chlorinated water solutions and were especially less inclined to accept chlorinated water as drinking water delivered at the tap. These results highlight the importance of tap water representation, beyond chlorine sensitivity and flavour perception, in water consumption and choices. Since very different participants were tested in terms of their water consumption habits, these findings may be extended to the French population. However, it would be interesting to perform similar studies in other countries in order to evaluate the influence of chlorine exposition level and other cognitive factors.
3. Publication 2:

**Trigeminal perception is involved in chlorine flavour perception and could account for Tap water rejection**

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1. Introduction

Chlorine flavour constitutes one of the major complaints addressed against tap water (Chotard 2008; Suffet *et al.* 1996; Hudon *et al.* 1991). Nevertheless, chlorine addition is a necessity for tap water suppliers to maintain a high bacteriological quality throughout the water-supply network. Most often, chlorination treatment is ensured by gaseous chlorine addition in water (Connell 1996). Chlorine\(^1\) (Cl\(_2\)) immediately reacts with water to form hypochlorous acid which is present both in its associated form (HOCl) and dissociated form (ClO\(^-\)) at the tap water pH value (6-8; Doré 1989). pKa of this acid is 7.5. Below this value, the quantity of HOCl increases whereas the quantity of ClO\(^-\) decreases.

\(^1\) At the treatment plant, chlorine gas (Cl\(_2\)) immediately reacts with water to form hypochlorous acid. As a consequence, the term chlorine cannot be used for the stimulus as far as chlorine in water is concerned. In this publication, the term chlorine flavour will be used only to describe the perception elicited by hypochlorous acid and it will be mentioned whether it is the associated (HOCl) or dissociated form (ClO\(^-\)).
Bryan, Kuzminiski et al. (1973) who measured chlorine flavour thresholds showed that chlorine flavour threshold decreases according to pH. Thus, an increase of the volatile associated form (HOCl) is associated with an apparent threshold decrease. However, this cannot be attributed to an increase in sensitivity since the mechanism only relies on the increase in stimulus concentration. Nevertheless, these data suggest that the volatile associated form, i.e. HOCl, is responsible of the sensory perception. Perception of chlorine flavour in water has rarely been studied in depth. Mackey et al. (2004a) have studied public perception of chlorine flavour in drinking water. They did not find a link between chlorine flavour thresholds and tap water consumption habits. In a previous study (Puget et al. 2010), we consolidated this result, since we did not find a significant difference in chlorine flavour perception (i.e. detection threshold and intensity) between tap water consumers and non-consumers. However, we evidenced a significant difference between these two groups in terms of preference and acceptability for solutions realised with increasing chlorine concentrations. These findings have suggested that, beyond chlorine sensitivity and flavour perception, tap water representations may account for water consumption behaviours and choices. In the same study (Puget, Beno et al. 2010), we also observed a relatively atypical profile of the stimulus-response curve. Perceived odour intensity usually increases as a monotonic function of the chemical stimulus concentration (Chastrette, Thomas-Danguin et al. 1998). In contrast, chlorine flavour intensity was found to increase between the detection threshold (0.14 mg/L free chlorine Cl\textsubscript{2} equivalent\textsuperscript{2}) and 0.3 mg/L Cl\textsubscript{2}; a plateau was observed between 0.3 and 3 mg/L Cl\textsubscript{2} then a sharp increase in intensity was noted between 3 and 10 mg/L Cl\textsubscript{2} (Puget, Beno et al. 2010). These results suggested that at least two sensory mechanisms could be involved in chlorine flavour perception. Indeed, Flavour is defined as a sensory percept which relies on the functional integration of information transmitted by the chemical senses: olfaction, gustation, oral and nasal somatosensory inputs (Small and Prescott 2005; Hummel 2008; Verhagen and Engelen 2006). The mechanisms implied in chlorine flavour perception remain largely unexplored. If HOCl is likely the actual chemical stimulus as suggested by the data reported by Bryan, Kuzminiski et al.

\textsuperscript{2} In this publication such as in the whole manuscript, concentration of hypochlorous acid in water are free chlorine expressed in mg/L Cl\textsubscript{2} equivalent. This will be written mg/L Cl\textsubscript{2}. 

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It is not known whether volatile HOCl could activate both olfactory and nasal trigeminal systems. Additionally, once in the mouth, chlorine in water can activate both gustatory and oral trigeminal systems. Even if no difference in sensitivity could be observed previously between tap water consumers and non-consumers for chlorine flavour threshold, it cannot be excluded that differences in one chemical sense sensitivity exists and can therefore account for variations in preferences and acceptability observed in relation to consumption habits.

It is possible to perform independent measurements of the olfactory, gustatory and somatosensory activation threshold. Phenomena occurring in nose or in mouth can be easily distinguished respectively through retronasal tasting with or without a nose clip. In fact, the use of nose clip prevents air flow movement conveying volatile compounds retronasally to the olfactory epithelium. Once in the mouth or in the nose, it is more difficult to distinguish taste and smell from trigeminal perception.

Indeed, trigeminal and gustatory systems are intimately associated in mouth. Most of the fibers entering the fungiform papillae are trigeminal (Farbman and Hellekant 1978) and interactions between gustatory and somatosensory nerve exist from the periphery to the brain (Faurion 2004). As a consequence, it is not straightforward to distinguish between the taste and the chemosensory perception elicited by a compound. Conversely, several methods were proposed to distinguish between odour and nasal irritation perception. In 2002, Shusterman reviewed the different methods developed to assess trigeminal function depending on whether physiological or perceptual responses are registered. The authors especially reviewed methods distinguishing between odour and nasal irritation perception. In 2002, Shusterman reviewed the different methods developed to assess trigeminal function depending on whether physiological or perceptual responses are registered. The authors especially reviewed methods distinguishing between odour and nasal irritation perception. The first method consisted to ask subjects to rate irritation using specific instructions that focalise subjects’ attention on trigeminal sensations. However, this method is sensitive to confusion between odour and irritation and has been judged less objective. Indeed, subjects’ responses could integrate cognitive aspects of the perception (Frasnelli and Hummel 2005).

Other methods compared nasal detection threshold in normosmic and in anosmic subjects. Detection threshold obtained with a normosmic panel is assumed to be the olfactory threshold whereas detection threshold measured in anosmics is supposed to be the trigeminal threshold. These studies have been especially used to determine properties of irritants such as the ability of the trigeminal system to detect series of chemicals (Cometto-Muniz et al. 2005; Cometto-Muniz et al. 1998; Cometto-Muniz et al. 1999). Anosmic subjects were found to be less sensitive than normosmic ones to trigeminal stimulation due to the lack of central nervous interaction between these two sensory systems (Frasnelli and Hummel 2007; Hummel et al. 2003). Iannilli et al. (2007) also demonstrated that activation pattern in response to a trigeminal compound such as CO₂ were different in both populations.
A third methodology based on nasal lateralization thresholds is more and more often used to estimate trigeminal sensitivity. Indeed trigeminal thresholds can be inferred from lateralization thresholds obtained from individuals with normal olfactory capability. The lateralization procedure relies on the possibility for human subjects to localize the nostril in which a trigeminal volatile compound is delivered. Such localization is not possible for pure olfactory compounds or when the odorant concentration is too low to activate the trigeminal system (Kobal et al. 1989). This method which has been validated through studies including electrophysiological measurements (Cometto-Muniz and Cain 1997; Stuck et al. 2006; Wysocki et al. 2003) was the one retained in the present experiment to estimate trigeminal thresholds.

Several published data (e.g. Doty 1975; Doty et al. 1978) indicated that olfaction is most often activated at lower chemical stimulus concentrations as compared to the trigeminal system. Such a difference in activation thresholds could account for an atypical profile of the stimulus-response curve. Indeed, the activation of the olfactory system at low concentration could lead to an increase of perceived intensity when concentration increases. At higher concentrations, the activation of the trigeminal system could lead to a modification of the perceived stimulus intensity, which may result in a modification of the stimulus-response curve slope. In this paper, we tested this hypothesis and set out to measure olfactory and trigeminal detection thresholds for chlorinated water solutions. We also measured an in mouth detection score at a high chlorine concentration. This last measure especially aimed to assess the putative activation of the gustatory system. All measurements were performed for a group of tap water consumers and a group of non-consumers since we wanted to know whether sensitivity differences in one of the chemical senses could be evidenced and therefore account for variations in preferences and acceptability observed in relation to tap water consumption habits (Puget, Beno et al. 2010).

2. Materials and methods

All experiments were performed in a room dedicated to olfactometry following HACCP and Research Quality Insurance Standards. Consumers’ responses were collected using software dedicated to sensory analysis (FIZZ, Biosystèmes, Couternon, France).

2.1. Consumers

Two hundreds consumers randomly selected from the Dijon area were first contacted for an inquiry on their water consumption and general food habits. On the basis of their responses, 72 out 200 were invited to participate to sensory deficiency screening tests and to a more extensive questionnaire on their drinking water habits. Screening tests consisted in the European test of olfactory capabilities (ETOC, Thomas-Danguin et al., 2003), a test to
evaluate subjects ability to rank six chlorine solutions with increasing sodium hypochlorite concentrations and a mental concentration test (Bourdon Test, Lesschaeve, 1997). This selection was done in order to avoid highly sensory deprived subjects and to be sure to include either exclusive tap water consumers or exclusive tap water non-consumers in two dedicated groups. Subjects were also selected for their availability during the whole testing sessions. A total of 40 out of 72 consumers participated to the study. Only one consumer was excluded because of his results to the screening tests (low score at ETOC).

On the one hand, consumers included in the tap water consumer group were people who daily drank chlorinated tap water without using any device or process to reduce chlorine flavour. Following the French standard concentration, these consumers received, at tap, a chlorine concentration between 0.1 and 0.3 mg/L Cl₂. On the other hand, consumers included in the tap water non-consumer group were people who did not drink tap water and declared themselves to be exclusive bottled water consumers.

Twenty tap water consumers (9 women, 11 men) with a mean age of 38 ± 11 years and 20 tap water non-consumers (14 women, 6 men) with a mean age of 43 ± 14 years participated in the measurements of hypochlorous acid olfactory thresholds.

Due to a problem in the data registration, in-mouth detection score were obtained for 39 participants: for this measurement, the tap water non-consumers group contained only 19 consumers (13 women, 6 men).

Only 32 out of the previous 40 consumers took part in the measurement of trigeminal lateralization thresholds: 15 tap water consumers (9 women, 42 ± 13 years old) and 17 non-consumers (8 women, 39 ± 11 years old). The participants signed an informed consent form but the aim of the experiment was not revealed. They were asked not to modify their water consumption during the study and to avoid smoking, drinking and eating at least one hour before each session and to avoid using perfume the day of the test. Subjects were paid for their participation. Each consumer participated in five sensory sessions; four were dedicated to trigeminal threshold measurements and one to olfactory threshold measurements and in-mouth detection score.

It is important to note the subjects are the same as recently in a previous manuscript by Puget, Beno et al. (2010).

2.2. Trigeminal threshold measurements

2.2.1. Stimuli and delivery apparatus

Along the water network, chlorination is mainly performed by addition of chlorine gas. This procedure aimed to form hypochlorous acid (ClO'/HOCl; pKₐ=7.5). This compound can also
be produced using sodium hypochlorite solution (NaOCl ~15%, RECTAPUR, VWR international, France).

For nasal trigeminal threshold measurements, Phenyl ethyl alcohol (PEA, SAFC; ≥ 99%) and 1-butanol (BUT, Sigma; 98.7%) were respectively used as pure olfactory and trigeminal controls (Doty, Brugger et al. 1978; Wysocki, Cowart et al. 2003).

A computer-controlled olfactometer based on air dilution birhinal olfactometry (OM4b; Burghart, Wedel, Germany) was used to deliver stimulants to the left or the right nostril. The air was humidified and temperature stabilized at 37°C at the olfactometer’s outlet. The total airflow from each outlet was kept constant at 6.0 L/min. Stimulus duration was 250 ms, the inter-stimulus interval (ISI) varied randomly between 30 and 40s. 1-Butanol and phenyl ethyl alcohol were set pure in a dedicated olfactometer chamber. A Sodium Hypochlorite solution (120 g/L Cl₂) was introduced in a third chamber. To avoid solution depletion, sodium hypochlorite solution was replaced between each session. Within the olfactometer, purified dry air went through the liquid to produce odorized air. This odorized air was diluted with humidified odourless air at the outlet of the olfactometer. Four stimulus flow / total air flow ratio were used for 1-butanol (0.3%; 0.9%; 2.6%; and 7.4%) and sodium hypochlorite (10%; 21%; 46%; and 100%). Phenyl ethyl alcohol was used at a 7.4% fixed ratio. When one nostril was stimulated using one of these ratios in the total air flow (6.0 L/min), the other one received odourless air (control) with the same total air flow (6.0 L/min). Stimulations were delivered through tubing terminating in a nosepiece inserted into the subjects' nostrils. They were not presented in synchrony with breathing. During stimulation periods, subjects performed the velopharyngeal closure technique in order to restrict breathing through the mouth only.

The concentrations of 1-butanol and phenyl ethyl alcohol were measured at the outlet of the olfactometer using gas chromatography equipped with a flame ionization detector and calibration curves. Concentrations of hypochlorous acid were measured by collection of volatile in sulfamic acid solution and on iodometric measurements of chlorine using a specific electrode (INRS 2006; Ku Revised 1991).

2.2.2. Sensory procedure

Each consumer participated in four sensory sessions. The first one was a 1-hour session which aimed to familiarise subjects with the apparatus, the velopharyngeal closure and the lateralization task. Subjects were trained to velopharyngeal closure using a flow-meter measuring the flow related to breathe in one of the subject’s nostril. Subjects could visualise on a computer screen that they could breathe by the mouth only and therefore isolate the nasal cavity from the rest of the respiratory tract.
Three 1H30 sessions were dedicated to measurements. 1-butanol lateralization thresholds were measured first, followed by the hypochlorous acid threshold measurement. Finally, lateralization scores were performed for Phenyl Ethyl Alcohol at a single fixed concentration. Lateralization threshold were measured according to the constant stimuli procedure (AFNOR 2002). This procedure allows psychometric function modelling on the basis of detection probability measurement for increasing stimulus concentrations. In our experiment, the detection probability was measured using a 2 Alternative forced choice procedure (2-AFC). In this test, stimulations were randomly presented in the right or the left nostril and consumers had to decide which one was stimulated. For 1-Butanol and Hypochlorous acid, each consumer performed 32 tests (n=32) at each of the 4 concentration levels. For each threshold, 128 stimulations were performed. They were divided into 8 blocks containing 16 stimulations. Presentation orders were equilibrated in each block.

For Phenyl ethyl alcohol, a lateralization score at a single concentration was performed using 32 tests (n=32) presented in 2 blocks of 16 stimulations.

Measurement sessions began with a short training during which they lateralized few stimulations of 1-butanol and received a feed-back on the correct answer. Then, 6 blocks were evaluated by session with a short break (1min) between each. A 15 min break was performed after 3 blocks. In the first session, 6 blocks of 1-butanol were evaluated. In the second one, the 2 remaining blocks of 1-butanol and 4 blocks for hypochlorous acid were evaluated. In the last session, 4 blocks were dedicated to hypochlorous acid evaluation and 2 for phenyl ethyl alcohol.

2.3. Olfactory threshold measurements

2.3.1. Stimuli and delivery apparatus

A dynamic olfactometer (AC'SCENT® International Olfactometer; St. Croix Sensory, Inc., U.S.A.) was used to estimate chlorine olfactory threshold. This olfactometer is based on the dilution of an odorous phase, contained in a Teflon® bag, by an odourless air flow according to specific dilution ratios. The odorous phase was obtained after 12-hour equilibration of 20 mL of Sodium hypochlorite solution (NaOCl ~15%, RECTAPUR, VWR international, France) with 11L pure nitrogen into a Teflon bag (49*49 cm, 20 L capacity, equipped with a Teflon-connector; Interchim France). Chlorine concentration in the odorous phase contained in the bag, was measured using the same methodology as for trigeminal threshold measurements. This olfactometer allows stimulus presentation following 3-alternative forced choice procedure and is dedicated to olfactory threshold measurements.
2.3.2. Sensory procedure

A one-hour session was dedicated to estimate individual olfactory thresholds according to the constant stimuli procedure (AFNOR 2002). Within this one-hour session, a best estimated threshold (BET) was first measured for every subject in order to determine a target range of concentrations to be used in the constant stimuli procedure. Six stimulus dilution ratios, around BET, were used for the precise threshold estimation. Thus, six 3-AFC (n=6) were performed for each of the 6 stimulus dilution ratios. The results were used to build a psychometric function and to determine thresholds.

2.4. In-Mouth detection score measurements

2.4.1. Stimulus delivery

For in-mouth detection score, chlorinated water samples were obtained by adding sodium hypochlorite (NaOCl ~15%, RECTAPUR, VWR international, France) to Evian water (La Bourgogne, Dijon, France). The chlorine concentration in the samples was 3 mg/L Cl₂. Since sodium hypochlorite solutions provide free chlorine but also sodium ions which may contribute to taste, control solutions used in discrimination tests needed to be compensated for sodium (Lugaz et al. 2002). Control solutions were prepared by adding sodium chloride (NaCl, Jera, France) to reach the same sodium content as in the chlorine solutions, namely 4.95 mg/L. Because of chlorine’s high volatility and degradation by sunlight (UV), chlorinated solutions were prepared daily and stored until tasting (8 hours maximum) in brown glass 500 mL flasks equipped with brown glass stoppers. Before use, flasks and stoppers were heated at 400°C for 2 hours and then 3 times rinsed with a sodium hypochlorite solution (1%) in order to minimize chlorine demand due to residual compounds which could remain on the glass. As a consequence, the expected chlorine content of the delivered samples was controlled by free and total chorine measurements. In the absence of chlorine demand these parameters are assumed to be close to the expected value. Both parameters were controlled daily in each flask just after solution preparation and at the end of each sensory session. These controls were performed through a procedure (pocket colorimeter II, Hach Lange) adapted from the DPD protocol for spectrophotometry (APHA-AWWA-WEF 1998).

For sensory tasting, samples of 10 mL were delivered in plastic glasses coded with a three digit number. During the tasting sessions, consumers were requested to wear a nose piece in order to block air flow movements in the nasal cavity which avoid nasal stimulation.

2.4.2. Sensory procedure
Prior to the experiment, a pilot study was conducted with an internal panel of 23 subjects performing one 3-AFC test at 3 mg/L Cl$_2$. Since no significant in-mouth detection of Hypochlorous acid (HOCl/ClO$^-$) was found, we chose not replace the measure of in-mouth detection thresholds by only a detection score at this concentration (3 mg/L). This measurement was performed at the end of the one-hour session dedicated to the olfactory threshold measurement, after a 10-minutes break. Each consumer group performed four 3-AFC at 3mg/L Cl$_2$ (n=4). This number of tests was chosen to be consistent with the number of repetitions used in a previous experiment (Puget, Beno et al., 2010). The consumers had to wait at least 140 sec between each trial and were instructed to rinse their mouth with Evian water during this inter-trial interval.

2.5. Data analysis

All statistical analyses were performed using SAS release 9.1.3 (SAS Institute Inc., Cary, NC).

2.5.1. PEA Lateralization scores and hypochlorous acid in mouth detection scores

For PEA lateralization thresholds, 32 consumers performed 32 2-AFC tests. First, the actual number of correct answers of each assessor was compared with the minimum number of correct responses required for a correct localization of PEA. This minimum number of correct responses was obtained using a SAS macro BINRISK (Schlisch 1993).

Following the strategy proposed by Kunert and Meyners (1999) to analyse responses of discrimination tests with replications, the actual distribution of correct answers of the assessors was compared to the theoretical binomial distribution ($B_{(p=1/2; n=32)}$). This comparison was performed using a Fischer exact test and allowed to determine whether or not PEA was in mean localised by the whole panel.

For in-mouth detection score, 39 consumers performed four 3-AFC. Due to the weak number of repetitions, responses were not analysed at individual level. To determine whether consumers detected or not chlorine in-mouth, the actual distribution of correct answers was compared to the binomial distribution ($B_{(p=1/3; n=4)}$).

2.5.2. Trigeminal and olfactory thresholds

For olfactory thresholds, 40 consumers performed 6 3-AFC tests (n=6, p=1/3) at 6 concentration levels whereas 32 consumers performed 32 2-AFC tests at 4 concentration levels (n=32, p=1/2), for trigeminal thresholds. Correct answers obtained in alternative forced choice tests were analysed to build a psychometric function and to determine thresholds.
according to the modelling proposed in (Puget, Beno et al. 2010). This modelling follows a
global approach in which (i) the number of correct answers is assumed to follow a binomial
law \(B(p=1/3; n=6)\) for olfactory thresholds and \(B(p=1/2; n=32)\) for trigeminal threshold, (ii) the logit
of the probability of detection is assumed to be linearly related to the logarithm of the
concentration, and (iii) each subject’s sensitivity to the stimulus is assumed to be randomly
distributed, according to a normal distribution.
Since our aim was to compare differences between the two groups, two models were built
with different slopes (\(\alpha\)). The first one was used to estimate the mean threshold for the whole
set of consumers. This model assumed that the slope \(\alpha\) is the same for the whole consumer
set. On the opposite, the second model took into account a putative sensitivity difference
between the two groups of panellists and assumed that both groups had different slopes,
\(\alpha_{TWC}\) for tap water consumers and \(\alpha_{TWC}\) for non-consumers.
Therefore, testing the difference between the two groups means testing the significance of
\(\Delta\alpha (\alpha_{TWC} - \alpha_{TWC})\). This was achieved by comparison of the two models (model 1 and model 2),
through a nested model comparison using likelihood ratio tests. This comparison aimed to
determine whether model 2 better fits the data than model 1. In other words, this comparison
allowed us to determine whether the sensory difference between tap water consumers and
non-consumers was statistically significant.

3. Results

3.1. Trigeminal thresholds

PEA lateralization score and 1-butanol lateralization threshold were used as controls to
validate thresholds obtained for Hypochlorous Acid.

3.1.1. PEA Lateralization score

PEA lateralization score were measured with 32 consumers following a 2-AFC procedure
including 32 trials: each consumer had to decide which nostril was stimulated. The number
of correct answers follows a binomial distribution probability (\(n=32; p=1/2\)). Therefore, for a risk
\(\alpha=5\%\), 22 is the minimum number of correct answers to decide that a consumer actually
lateralized PEA (Figure 1). Four out of 32 consumers obtained scores higher than this critical
value. At the group level, we used data treatment proposed by Kunert and Meyners (1999)
and we assumed that consumers could belong to two different populations, those who are
able to lateralize PEA and those who are not. As a consequence, we have simulated the
performance of 32 assessors under the hypothesis of absence of lateralization (binomial
distribution \(n=32; p=1/2\)) and obtained a theoretical distribution. Then, we compared this
theoretical distribution to the actual distribution of correct answers obtained for PEA lateralization (Figure 1). Kunert and Meyners (1999) performed the comparison between the two distributions using a $\chi^2$-goodness-of-fit-test. However, due to the rather low number of consumers involved in the experiment, we performed this comparison using a 2-tailed Fischer Exact Test which was not significant (p= 0.78; FET). This result indicated that the observed distribution was not significantly different from the theoretical distribution. Therefore, at the group level, it could not be concluded that subjects were able to correctly localize the nostril stimulated with PEA. Despite consumers declared to clearly perceive PEA odour, the panel failed to lateralize PEA at the concentration tested 1.4 ppm (Vol/Vol). At this concentration level, PEA may not activate the trigeminal system.

![Figure 1: Correct answers distribution obtained for the PEA lateralization](image)

This figure presents distribution of correct answers obtained for PEA lateralization. Thus, the number of subjects who gave a definite number of correct answers is represented in absolute frequency. The red line represents the minimal number of correct answer (22) to decide that a consumer actually lateralizes PEA. 4 consumers were in this case. The actual correct answers distribution obtained from the whole consumer set is represented by the black bars (■) whereas the theoretical binomial distribution ($B_{\left(n=32 ; p=1/2\right)}$) is represented by the grey bars (■). The statistical comparison between these two distributions through 2-tailed Fischer Exact Test was not significant (p= 0.78; FET) indicating that the whole set of consumers not lateralize PEA.

### 3.1.2. 1-Butanol lateralization thresholds

The results revealed that consumers were able to localise 1-butanol. The modelling allowed to estimate the mean lateralization threshold at 521 ppm (vol/vol, SEM=66) for the tap water consumer group. The mean lateralization threshold was estimated at 397 ppm (vol/vol, SEM=49) for the other group of consumers. However, the comparison between the two models (nested model likelihood ratio test) revealed no significant difference ($\chi^2_{\left(1, N=32\right)}=2.5$, p=0.11) which demonstrated that the data were equally well-described by both models. As a consequence, 1-butanol lateralization threshold should not be considered as significantly different between the two groups of consumers. Therefore, the mean 1-butanol lateralization threshold was estimated through a model which did not take into
account a difference between the two groups of consumers and the mean lateralization threshold was estimated at 458 ppm (vol/vol, SEM=41) for the whole set of consumers. Individual thresholds were also estimated and presented Figure 2.

![Figure 2: 1-Butanol lateralization thresholds](image)

1-butanol lateralization thresholds distribution for tap water non-consumers (n=17), tap water consumers (n=15) and the whole consumer set (n=32). Tap water consumers and Tap water non-consumers’ individual (□) and mean (—) thresholds were estimated using a model which assumed a difference in threshold for both consumer groups. Individual (□) and mean thresholds (—) for the whole set of consumers were estimated using a model which assumed no difference between the two groups.

Mean values (—) are expressed in ppm (Vol/Vol). NS indicated that the 1-Butanol mean lateralization thresholds should not be considered as significantly different between the two groups of consumers. This was determined by sub-model comparison through likelihood ratio tests.

### 3.1.3. Hypochlorous Acid lateralization threshold

Hypochlorous Acid lateralization threshold was estimated using the same procedure as for 1-butanol. The results revealed that consumers were able to localise the nostril stimulated with hypochlorous acid. The mean hypochlorous acid lateralization threshold was estimated at 38.5 ppb (vol/vol, SEM=2.5) for the tap water consumer group and at 40 ppb (vol/vol, SEM=2.8) for the non-consumer group. The comparison between the two models revealed no significant difference ($\chi^2_{[1, N=32]}=0.2$, p=0.7, nested model likelihood ratio test) which demonstrated that the data were equally well-described by both models. As a consequence, hypochlorous acid lateralization threshold was estimated using the model gathering all consumers’ data since the difference between lateralization thresholds of the two groups should not be considered as significant.

Therefore, the hypochlorous acid mean lateralization threshold was estimated at 39 ppb (vol/vol, SEM=1.9) for the whole set of consumers. Individual thresholds were also estimated and reported on Figure 3.
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Figure 3: Hypochlorous acid lateralization thresholds

Hypochlorous acid lateralization thresholds distribution for tap water non-consumers (n=17), tap water consumers (n=15) and the whole consumer set (n=32). Tap water consumers and Tap water non-consumers’ individual (□) and mean (—) thresholds were estimated using a model which assumed a difference in threshold of the two consumer groups. Individual (□) and mean thresholds (—) for the whole set of consumers were estimated using a model which assumed no differences between the two groups. Mean values (—) are expressed in ppb (Vol/Vol). NS indicated that the Hypochlorous acid mean lateralization thresholds should not be considered as significantly different between the two groups of consumers. This was determined by sub-model comparison through likelihood ratio tests.

3.2. Hypochlorous Acid Olfactory thresholds

As aforementioned, Hypochlorous acid olfactory threshold was estimated through the same statistical procedure as the lateralization thresholds. For the tap water consumers’ group, this detection threshold was estimated at 0.49 ppb (vol/vol, SEM=0.08) and at 0.49 ppb (vol/vol, SEM=0.08) for the non consumers’ group. The difference between detection thresholds of the two groups should not be considered as significantly different ($\chi^2(1, N=32)=0, p=1$, nested model likelihood ratio test). Hypochlorous acid threshold was estimated at 0.49 ppb (vol/vol, SEM=0.06) for the whole set of consumers. Individual thresholds were also estimated and reported on Figure 4. Inter-individual variations are greater than the one observed for lateralization thresholds.
Figure 4: Hypochlorous acid olfactory thresholds

Hypochlorous acid olfactory thresholds distribution for tap water non-consumers (n=20), tap water consumers (n=20) and the whole consumer set (n=40). Tap water consumers and Tap water non-consumers’ individual (□) and mean (—) thresholds were estimated using a model which assumed a difference in threshold of the two consumer groups. Individual (□) and mean thresholds (—) for the whole set of consumers were estimated using a model which assumed no differences between the two groups. Mean values (—) are expressed in ppb (Vol/Vol). NS indicated that the Hypochlorous acid mean olfactory thresholds should not be considered as significantly different between the two groups of consumers. This was determined by sub-model comparison through likelihood ratio tests.

3.3. In-mouth detection score

For in-mouth hypochlorous acid detection score, 39 consumers performed four 3-AFC tests. Since, each consumers performed a small number of tests (n=4), data were not analysed at the individual level but only at the group level following the strategy proposed by Kunert and Meyners (1999). The actual distribution of correct answers of the assessors was compared to the theoretical binomial distribution (B\((p=1/3; n=4)\)). This comparison was performed using a Fischer exact test (FET) and was not significant (p= 0.40; FET; Figure 5). This means that the whole set of consumer was not able to detect hypochlorous acid at 3 mg/L Cl₂ on the basis of their in-mouth perception (gustation and/or somatosensory sensation).
Figure 5: Correct answers distribution obtained for Hypochlorous acid in-mouth detection

This figure presents the distribution of correct answers obtained for Hypochlorous acid in-mouth detection measurements. Thus, the number of subjects giving a definite number of correct answers is represented in absolute frequency.

The actual correct answers distribution obtained from the whole consumer set (n=39) is represented by the black bars (■) whereas the theoretical binomial distribution (B(n=39; p=1/3)) is represented by the grey bars (▲). The statistical comparison between these two distributions through 2-tailed Fischer Exact Test was not significant (p=0.40; FET) indicating that the whole set of consumers did not detect hypochlorous acid on the basis of their in-mouth perception.

4. Discussion

One of the issues of this study was to determine the sensory mechanism involved in chlorine flavour detection. Our results indicated that the whole set of consumers failed to significantly detect hypochlorous acid on the basis of their in-mouth perception. However, we evidenced that hypochlorous acid could activate both the olfactory and nasal trigeminal systems. The olfactory threshold and lateralization thresholds were respectively 0.49 and 39 ppb for the whole consumer set. Additionally, PEA lateralization score and 1-butanol trigeminal threshold were measured as control to validate hypochlorous acid lateralization threshold.

In the present study, we chose to infer trigeminal thresholds from lateralization thresholds measurements (Wysocki et al. 2003; Kobal et al. 1989). This methodology was used to estimate both individual and panel lateralization thresholds for 1-butanol. This volatile compound was used as a positive control since it is known to elicit medium trigeminal properties (Wysocki et al. 2003; Doty et al. 1978; Jacquot et al. 2004). This control allowed us to verify the ability of the subjects to perform the lateralization task. We obtained a mean lateralization threshold of 485 ppm. This value is a little lower than those measured by Cometto-Muniz et al. (2000) who compared two stimulus-delivery systems for measurement of nasal pungency threshold. These authors estimated 1-butanol threshold between 900 and 4000 ppm depending on whether stimulus was delivered respectively using glass vessels or
squeeze bottles. This difference could be explained by the precision of the stimulus delivery used but also by the fact that in our study, thresholds were measured in normosmic subjects whereas Cometto-Muniz et al. (2000) performed their study with anosmic subjects. Indeed, Cometto-Muniz et al. (1998) observed that anosmics were less sensitive than normosmic subjects. This difference has been confirmed in numerous studies (see Shusterman 2002 for review). Hummel et al. (2003) showed an improvement of lateralization capacities depending on the duration of the olfactory impairment. These observations were confirmed by Frasnelli and Hummel (2007) who reported a decrease in anosmic trigeminal sensitivity due to the loss of central interaction between olfactory and trigeminal systems. Iannilli et al. (2007) also demonstrated a difference in fMRI activation patterns between anosmic and normosmic subjects.

We also estimated the lateralization score for Phenyl Ethyl Alcohol (PEA). This volatile compound was used as a negative control since it is considered as a pure odorant and several studies demonstrated it can not be localized (Doty, Brugger et al. 1978; Wysocki, Cowart et al. 2003; Frasnelli et al. 2009). We measured a lateralization score at quite a high PEA concentration (1.4 ppm vol/vol) and concluded that lateralization did not occur at the panel level (n=32). Nevertheless, 4 out of 32 subjects were able to localize PEA significantly. Some studies also reported such observation (Doty, Brugger et al. 1978; Porter et al. 2005). However, data analysis indicated that these correct localisations could occur by chance and PEA should not be considered as localized by consumers in our study.

Since the results we obtained with control stimuli are in accordance with previously reported data, we considered our methodology as valid for the measurement of sensory characteristics of hypochlorous acid. It is important to note that the associated form (HOCl) of hypochlorous acid is volatile, whereas the dissociated form (ClO\(^{-}\)) is non-volatile. As a consequence, as far as orthonasal measurements are concerned, HOCl has to be considered as the sensory stimulus. When in-mouth measurements are concerned, both forms associated and dissociated may activate taste or somatosensory receptors. Our results demonstrated that HOCl can be lateralized by the group of consumers. The mean lateralization threshold for the consumers’ panel (n=32) was 39 ppb (vol/vol) which is quite a low concentration value. Using the Henry’s law constant (Blatchley et al. 1992), we estimated that this hypochlorous acid lateralization threshold can be obtained with chlorine solution concentration of 4.1 mg/L Cl\(_2\). Despite of the use of constant stimuli procedure which offers good individual threshold estimation (Wise et al. 2008), our results did not evidence any difference in hypochlorous acid lateralization threshold between tap water consumers and non-consumers. Hypochlorous acid olfactory detection threshold was estimated at 0.49 ppb (vol/vol) for the same panel of consumers. We did not evidence any significant difference between tap water consumers and non-
consumers. This olfactory detection threshold is 10 times lower than the lateralization threshold (39 ppb) which is in line with knowledge on trigeminal perception (Doty 1975; Doty et al. 1978; Doty and Cometto-Muniz 2003). Using HOCl Henry’s law constant (Blatchley, Johnson et al. 1992), we calculated that the olfactory detection threshold can be reached in a water solution chlorinated at a concentration of 0.05 mg/L Cl₂.

When studying the chlorine flavour intensity, Puget, Beno et al. (2010) reported an atypical profile of the stimulus response curve (Chastrette, Thomas-Danguin et al. 1998). Indeed, chlorine flavour intensity was found to increase until 0.3 mg/L; a plateau was observed between 0.3 and 3 mg/L then a sharp increase in intensity was noted between 3 and 10 mg/L. We advocated that this shape of the stimulus response curve could rely on the activation of differentiated chemical senses, each one having a specific activation threshold. This hypothesis is reconcilable with the results obtained in the present study. Indeed, olfaction is likely activated by volatile HOCl at low concentration from an in-water chlorine concentration of 0.05 mg/L Cl₂. When the concentration reaches 0.3 mg/L, this could correspond to a saturation of the olfactory function. As a result, the stimulus response function followed a plateau until the stimulus concentration reaches the value of 4 mg/L Cl₂. At this concentration value, the trigeminal system could be activated and the stimulus response function was found to increase again. Flavour perception not only implied olfactory and trigeminal perception but also gustatory information which has to be considered. Our results showed no significant gustatory detection at 3 mg/L Cl₂. This suggests that hypochlorous acid (HOCl/ClO⁻) does not activate the gustatory system at least until this concentration. When taken together, these results confirmed that the associated form of hypochlorous acid (HOCl) is the chlorine flavour sensory stimulus as suggested by previous threshold measurement performed by Bryan, Kuzminiski et al. (1973).

In France, where the study was performed, the chlorine concentration in water at tap is supposed to fall between 0.1 and 0.3 mg/L (Journal Officiel 2001). Our present results indicated that, at these concentration levels, chlorine flavour perception relies only the activation of the olfactory function. Our data also highlighted that tap water consumers differed from non-consumers neither on their olfactory sensitivity to HOCl nor on their trigeminal sensitivity to this compound. Indeed, no significant differences were found between the two groups of consumers, neither on the olfactory dimension nor in the trigeminal nasal dimension of hypochlorous acid perception. Similarly, no differences were observed on flavour detection thresholds between the two consumer groups (Puget, Beno et al. 2010). As a consequence, it seems that the difference in hedonic ratings and acceptability previously observed between the two consumers groups (Puget, Beno et al. 2010) should not be linked with direct sensitivity differences.
5. Conclusion

In the present study, we proposed that chlorine flavour perception relies on olfactory activation at low concentration and trigeminal activation at higher concentrations. Our data also suggest that these perceptions are elicited by the volatile associated form of hypochlorous acid (HOCl). This study also showed that chlorine does not activate the gustatory system at concentrations reconcilable with those delivered at tap (<3 mg/L Cl₂). This study also confirmed that tap water consumers and non-consumers do not differ in their sensitivity to chlorine. This tend to confirm that the differences observed rely more probably on hedonic and acceptability differences as suggested by our previous study (Puget, Beno et al. 2010).
4. Partial discussion

The taste of water and especially chlorine flavour constitute one of the major complaints addressed against tap water. This has been highlighted through various surveys performed all over the world. However, very few studies explored the link between tap water consumption and sensory sensitivity to chlorine. In the same way, sensory mechanisms underlying chlorine flavour perception remained unknown. In such a context, our studies provide important outcomes.

We determined that chlorine flavour relies on the activation of the olfactory system at low chlorine concentration. The trigeminal nerve is also activated but at much higher concentrations. Our data also tend to confirm that the volatile form of hypochlorous acid, the sensory stimulus eliciting chlorine flavour perception, could be reconciled with those delivered at tap. Nevertheless, this remains to be confirmed through dedicated physicochemical analyses.

Our data did not evidence any significant sensitivity difference between tap water consumers and non-consumers. But tap water consumers were found to have a higher preference and were more inclined to accept chlorinated solutions than non-consumers. This suggests that chlorine flavour representations in tap water are determinant of the consumer behaviour. Indeed, several studies demonstrated that chlorine perception was associated with risk perception (Doria 2006; Hudon, Zayed et al. 1991; Jardine et al. 1999; Anadu and Harding 2000). Taken together, chlorine flavour seems to play in fine against tap water acceptability as a marker of safety representation. In such a context, it is important to remind that hypochlorous acid has a dual significance: food as far as tap water is concerned and non-food when bleach and cleaning are concerned.

Recent studies demonstrated that association processes could depend on the stimulation route (Bender et al. 2009). These authors demonstrated that separable signals are generated from the same food odour depending upon stimulation route. This phenomenon seems to be category specific. Indeed, this is true only for odour with food significance. In the same way, in a review on specificities of retronasal perception, Hummel (2008) clearly demonstrated that ortho and retronasal odour stimuli are differentially processed and produce different brain activation patterns. Retronasal route relies more on food odour processing whereas orthonasal route could also rely on non-food odours. These findings could have important implications as far as the results of the two studies presented in this chapter are concerned. It is likely that chlorine odour has a dual meaning. Therefore, the stimulation route could have
an important impact on the processing of this odour. It is important to keep in mind that olfactory and trigeminal thresholds, as measured in the present studies, were obtained through the orthonasal stimulation route. These thresholds were not found to be significantly different between the two groups of consumers. However, as far as chlorine flavour is concerned, the perception was not different between the two consumers group but one can observe on the stimulus-response curve (Figure 2) that chlorine flavour was perceived as more intense by tap water non-consumers at 3 and 10 mg/L which are concentrations reconcilable with trigeminal activation (Figure 2). This suggests that a difference between the two groups could exist but may depend on their retronasal sensitivity. This hypothesis could be consistent with the measurement performed by Heilmann and Hummel (2004) which demonstrated that thresholds can differ accordingly to the stimulation route.

![Figure 2: Chlorine flavour stimulus response curve (Puget, Beno et al. 2010)](image)

However, our data are not sufficient to validate this hypothesis. Thus, it would be interesting to further explore this point through dedicated experiments such as the comparison of chlorine flavour perception by tap water consumers and non-consumers via orthonasal and/or retronasal route.

To conclude, it is important to remind that we determined the sensory modalities involved in chlorine and their activation thresholds. This constitutes an essential outcome to elaborate a strategy to find masking compounds and interpret data presented in the following chapters.
Chapter 2. Water mineral matrix variations and their impact on chlorine flavour perception
Chapter II – Water mineral matrix variations and their impact on chlorine flavour perception – Context and strategy

One of the objectives of the PhD is to explore putative chlorine flavour perceptual neutralisation levers. This chapter aimed to present one approach which relies on the potency of mineral matrix constituents to mitigate chlorine flavour perception.

In the first chapter, we have determined the sensory modalities underlying chlorine flavour perception. Indeed, we demonstrated that hypochlorous acid is able to activate the olfactory sensory system at low concentrations and the trigeminal sensory system at concentrations higher than 4.1 mg/L. Since hypochlorous acid concentration does not usually exceed 0.3 mg/L in water delivered at tap, one can consider that the olfactory system only is activated during tap water consumption and supports the chlorine flavour perception.

Several sensory data have shown that minerals dissolved in water can be detected by the gustatory systems (e.g. Na\(^+\) cations which confer a salty taste; Murphy et al. 1981). Even if the sensitivity of the gustatory system to other minerals (e.g. Ca\(^{2+}\)) is less clear, it seems that water mineral content may be the support of water taste (Burlingame et al. 2007; Teillet et al. 2009a; Teillet et al. 2009b). Therefore, it is likely that dissolved minerals could activate the gustatory receptors located on the tongue. As a consequence, we postulate that perceptual interactions could occur during multisensory integration between chlorine odour and the taste elicited by minerals dissolved in water. These perceptual interactions may lead to a decrease of the chlorine flavour perception, at least for specific water mineral matrix.

Before to start the investigations on cross-modal interactions, it was a need to fix the determinants of drinking water taste. This has been done through three studies reported in Part I of this chapter. This part contains a short bibliographical review on components of water mineral matrix that could elicit a taste perception followed by three publications and a short summary of the results. On the basis of the result obtained, we investigated perceptual interactions between the perceptions of water mineral content (water taste) and hypochlorous acid (chlorine odour). The corresponding results are reported in Part II of this chapter which contains a bibliographical review on smell-taste interactions followed by two publications and a summary of the results.
1. Introduction

If drinking water is often defined as a colourless, tasteless and odourless product, it has been reported that the mineral content confers a taste to drinking water (Burlingame et al. 2007; Teillet et al. 2009a; Teillet et al. 2009b). Taste perception is mediated by taste receptor cells which are located on the tongue (edge and anterior dorsal part) but also on the soft palate, the pharynx and the larynx (Breslin and Huang 2006). These cells predominantly reside within the taste buds which are included into the papillae. Fungiform papillae are spread on the anterior two third of the tongue, foliate papillae are located on the lateral edge and circumvallate papillae on the posterior part of the tongue. Fungiform papillae contain from 0 to 15 taste buds whereas foliate and circumvallate papillae often contain dozens (Breslin and Huang 2006; Chandrashekar et al. 2006). Taste buds are ‘rosebuds’-shaped structures that contain about 60 to 120 cells. Four types of cells (I, II, III and IV) are enumerated and can be distinguished according to their functionalities. Type I and II cells have microvilli located in their apical part that are in direct contact with saliva containing dissolved soluble compounds able to activate them. It is unclear whether Type I cells are involved in signal detection or transduction. Some authors have hypothesized that these cells could play a secretory role or have a glia-like function within the taste bud. In the same way, type IV cells do not have a direct role in taste perception, they are considered as basal cells (Breslin and Huang 2006). On the opposite, type II and type III cells play a crucial role in taste perception.

Type II cells so called ‘receptor’ cells, possess the G-protein-couple receptors (GPCR) and are implied in bitter, sweet, and umami taste perception. However, there are some evidences that sweet, umami, bitter and sour (and, by extrapolation, salt) taste cells are segregated into non-overlapping populations expressing distinct receptors. This discovery leads some authors to support the hypothesis that labelled line model could explain taste coding (Chandrashekar et al. 2006). In such model, receptor cells are tuned to respond to single taste modalities (sweet, bitter, sour, salty or umami) and are innervated by individually tuned nerve fibres: 82% of these cells responded to only one taste stimulus (Tomchik et al. 2007).
However, data recently acquired on type III cells and cell to cell communication within the taste bud seems to invalidate the labelled line model.

It has been demonstrated that type III taste cells have prominent synapses with afferent nerve fibers. These cells thus called ‘synaptic cells’ were found to respond to acids, which elicit sour taste. The non-selective cation channel PKD2L1 is expressed exclusively in type III cells, where it has been hypothesized as the sour receptor. These cells are also known to express, Na+ channel (Roper 2007; Vandenbeuch and Kinnamon 2009). Additionally, some data evidence that type I cells and type II cells actually communicate. Tomchik et al. (2007) reported that in contrast to type II cells, type III cells are broadly tuned: 83% of these cells were found to respond to two or more different taste qualities. These data support the hypothesis of cell to cell communication within the taste bud (Huang et al. 2007). Type II cells which support a GPCR also produce ATP in response to a gustatory stimulation. ATP is able to directly activate afferent fibers but could also activate Type III cells (synaptic cells). Once activated, Type III cells produce serotonin (5HT) possibly at the synapse they made with sensory afferent fibers. When taken together, these data suggest that gustatory coding relies more on across-fibre activation patterns.

Beyond the few data available on taste cells, the mechanisms underlying taste coding and perception remain poorly understood and still debated for some of them. Mechanisms potentially involved in the perception of minerals dissolved in water are not exceptions. Murphy et al. (1981) systematically investigated the taste of 15 halide salts (LiCl, LiBr, LiI, NaCl, NaBr, NaI, KCl, KBr, KI, RbCl, RbBr, RbI,CsCl ,CsBr, CsI). In this study, subjects were asked to rate perceived intensity of sweet, sour, salty and bitter taste. The authors highlighted the importance of both cation and anion on taste perception of these salts which mainly elicit bitter and salty taste. Lighter cations Li+ and Na+ were rated as saltier. Nevertheless, weight of cation has no consistent effects on perceived saltiness. Lighter anions produced saltier tasting salts. Conversely, heavier cations and anions are associated with more bitter tasting salts. Heavier anions and cations are more incline to form ion pairs likely to activate bitter receptors.

Nowadays, cellular mechanisms underlying these observations are better understood thanks to electrophysiology and molecular biology. Thus, Na+ is responsible of the salty taste due to NaCl. One can distinguish two types of channels sensitive to Na+. Amiloride-sensitive channels constitute the first category (Roper 2007; Sugita 2006). These channels, apically localised, are the Epithelial Na+ Channels (ENaC) and are susceptible to be blocked by amiloride. Na+ concentration gradients between cell and saliva lead to a passive Na+ influx within the cell. This influx would depolarize and excite the taste receptor cell. Channels included in the second category are Amiloride-insensitive and consist in Vanilloid receptor-1 (VR1). Generally, VR1 are permeable to Na+, K+ and Ca2+ but relatively selective for Ca2+.
(\(P_{Ca}/P_{Na} = 10\)) (Roper 2007; Sugita 2006). These channels have a basolateral localisation. This localisation, if of high importance, explain the contribution of the Na\(^+\) contre-ion (the anion) to sodium salty taste. Thus, NaCl evokes a stronger saltiness than Na\(_2\)SO\(_4\). The explanation relies on the fact that, within the taste bud, apical cell-cell junctions are partially permeable to Na\(^+\) and Cl\(^-\). It represents a paracellular leakage for NaCl allowing the access of Na\(^+\) ions to the basolateral channel. On the opposite, large anions such as SO\(_4^{2-}\) do not pass through the tight paracellular spaces and reduce the Na\(^+\) access to basolateral channels (Elliott and Simon 1990; Ye et al. 1991; 1994). As far as K\(^+\) is concerned, this cation may activate not only amiloride-insensitive salt taste cells but also multiple taste receptor cells by eliciting membrane depolarization through various kinds of apically and basolaterally located K\(^+\) channels (Sugita 2006). These findings are in accordance with the observation done by Murphy et al. (1981).

In the context of this PhD project framework, it is also important to consider acid taste perception since acids have a taste. Beyond taste, acids could, though pH modulation, have an impact on the proportions of associated and dissociated forms of chlorine in water. Acids perception relies on the integration of several transduction pathways and implies different types of channels which are extra-cellular, intra-cellular, apical or basolateral. Some of these channels are especially dedicated to protons sensing such as Acid Sensing Ion Channels (ASIC) or Cyclic Nucleotide-gated ion (HCN) channels or aforementioned PKD2L1. However, ENaC which are involved in Na\(^+\) detection are likely to be permeable to H\(^+\). VR1 could be activated at pH lower than 5.9. Finally, as for salty taste, it has been demonstrated that anion could play a role in acid perception. Thus, acids, in their associated forms, are likely to penetrate within the cell and then be prone to dissociation leading to an acidification of the cytosol which is followed by an activation of the cell (Roper 2007; Sugita 2006).

After considering monovalent cations, we have to consider divalent cations such as Ca\(^{2+}\) and Mg\(^{2+}\). Schiffman and Erickson (1971) classified CaCl\(_2\) and MgCl\(_2\) as bitter–salty. MgSO\(_4\) and MgCl\(_2\) were used as bitter stimuli (Delwiche et al. 2000; Schiffman and Erickson 1971). However, several studies revealed that divalent salts have a complex taste. Thus, Tordoff (1996) demonstrated that CaCl\(_2\) was characterized as bitter, sour and sweet at 1 mM but bitter, salty and sour at 100 mM. Lawless et al. (2003) examined the taste profiles of CaCl\(_2\), MgCl\(_2\) and MgSO\(_4\) and reported that these divalent cation salts were characterized primarily by bitter taste, with additional sensations described such as salty, metallic, astringent, sour and sweet. Both studies also evidence the inhibitory impact of anions on perception of these divalent salts. This could be explained as for sodium salts, by the inability of larger anions to diffuse across paracellular junctions and into basolateral areas of taste receptor cell (Ye et al. 1991). The study conducted by McCaughey and Tordoff (2002) showed that magnesium or calcium deprived rats have distinct and specific appetites for these cations. This suggests
that calcium and magnesium can be distinguished. Data recently acquired using knock out mice suggest that T1R3, a receptor involved in sweet and umami detection, could also be a gustatory calcium-magnesium receptor (Tordoff et al. 2008). Gabriel et al. (2009) demonstrated that Calcium-sensing receptors (CaSR) usually signalling Ca\(^{2+}\) and Mg\(^{2+}\) in kidney are expressed in a subset of cells in circumvallate and foliate papillae. This suggests that this GPCR could also be involved in Ca\(^{2+}\) and Mg\(^{2+}\) taste. Once again, molecular mechanisms underlying taste of theses two divalent salts seem to be plural.

One of the important outcomes of psychophysical studies on divalent salts taste perception is methodological but has a consequence on the conception of taste categories. As explained by Faurion (1993), Henning (1916) defined taste space as a continuum of sensations arranged according to a tetrahedron; salty, bitter, sweet and acid being apex of this space. With years, only these tastes were kept as primary taste categories and many studies were conducted considering only these descriptors and studies regarding ions are not exceptions (Murphy et al. 1981; Tordoff 1996). Other primarily tastes are now admitted such as umami or more recently fat taste.

Due to the taste complexity of divalent salts, Yang and Lawless (2005) set out to better traduce sensation experienced by the subjects stimulated with such salts and used quantitative descriptive profiling to describe a series of divalent salts. This approach led to the use of a more extensive vocabulary and a more comprehensive description of sensation experience (e.g. metallic, astringency, after taste, mouthfeel, glutamate-like and spicy). In a latter study, Lim and Lawless (2005) but also Stevens et al. (2006) proposed to avoid as much as possible semantic cues to restituate sensory experience. To do so, a sorting task was used. This methodology is based on similarity judgement. In such experiment subject were asked to sort stimuli into groups having similar taste qualities.

In the field of water sensory evaluation, Meng and Suffet (1992) evaluated Flavour profiling data of water and reported difficulties to compare the meanings of descriptive terms because of inconsistent usage of qualitative descriptors by trained panellists. Based on this observation, Falahee and MacRae (1995; 1997) successfully applied multidimensional scaling analysis based on sorting or ranking procedure to describe qualitative variations occurring among drinking waters (distilled water, bottled water, tap water and blends of these waters.

More recently, Teillet et al. (2009b) also used a sorting task to assess the taste of drinking water. The authors assessed the sensory properties of a set of samples including bottled and tap waters. To do so, they compared conventional sensory profiling with other methods such as temporal dominance of sensations and free sorting task. Sensory profiling was found to be less discriminative as compared to free sorting. These authors also evidenced that the temporal dimension provided by temporal dominance of sensations is not essential to
describe perceptions elicited by drinking water. Drinking water taste was found to be largely determined by TDS (Total Dissolved Solids) of water. This is an important finding because TDS is a global variable combining both qualitative and quantitative information regarding water composition. These data especially suggest that water taste relies on the total amount of ions in water rather than ions nature and putative perceptual taste quality.

A physiological mechanism that could be implied in such a global perception has been propose and relies on sensory adaptation to saliva. Saliva constitutes a reference environment at which taste receptor cell are adapted. Saliva contains ions, mainly Na⁺, K⁺, Cl⁻ and HCO₃⁻, which are constantly at taste receptor cell's contact. Ions continuously stimulate these receptors, thus leading to an adaptation mechanism (Bartoshuk 1974; Matsuo 2000; Spielman 1990; McBurney and Pfaffmann 1963).

In 1992, Matsuo and Yamamoto recorded rats’ chorda tympani after the tongue was washed off. The authors registered responses to various solutions (NaCl, Sucrose, HCl, Quinine) and distilled water after adaptation to saliva, dialysed saliva but also distilled water. Results indicated that distilled water elicits a response when the tongue was adapted to saliva. Such a response was not observed after adaptation to distilled water or dialysed saliva that is saliva in which the ionic content has been removed. This result suggests that the taste elicited by distilled water (water taste) should be linked to the ion removal. In the same publication, the authors studied the effect of saliva ionic constituents on sensory responses. The authors demonstrated that ions in greater amount (Na⁺, K⁺, Cl⁻, et HCO₃⁻) had a significant activity on sensory response whereas ions found in lower amount (< 5 meq/L, i.e. Ca²⁺, Mg²⁺, F⁻, I⁻, H₂PO₄⁻) had no impact. Research studies conducted by Gilberson et al. (2002) suggested that beyond ion removal, the influx of water itself into taste cells could activate other signaling pathways leading to depolarization of the cell. Gilbertson et al. (2002) demonstrated that water influx during hypoosmotic stimulation caused cell swelling and subsequent activation of volume regulated anion (Cl⁻) channels. In a recent study, (Watson et al. 2007) evidenced that aquaporine is expressed in taste buds and that the AQ5 in particular could be involved in the gustatory response to water.

It appears that mechanisms implying adaptation to saliva or osmotic sensing could account for the results obtained by Teillet et al. (2009ᵃᵇ). However, as explained by Burlingame et al. (2007), anions and cations occurs in water as a multifaceted “soup” and more research is needed to understand their individual and combinatorial effects on perception but also on preference. Aforementioned studies conducted to elucidate mechanisms underlying taste perception cannot be used directly to understand determinants of drinking water taste but can help to set up hypotheses. Indeed, concentrations of ions used in studies on the gustatory system are not reconcilable with drinking water minerals low concentrations. Indeed, it has been demonstrated that water itself elicits a bitter taste. Therefore, at low
minerals concentrations, near detection threshold, confusions could occur between water
taste and taste elicited by minerals (Murphy et al. 1981; Bartoshuk 1974). Moreover, many
studies compared perceptual or electrophysiological responses elicited by single salts; but
drinking water always contains a mineral matrix made of a mixture of ions at different
concentrations. Thus, phenomena occurring during water consumption are difficult to
investigate.

Studies have been also conducted to evaluate the perception of tastants mixtures. As
reviewed by Schifferstein (2003), some of them aimed to evaluate substances eliciting similar
tasting whereas other aimed to determine the qualitative taste modification occurring when
substances with dissimilar taste are mixed. Some studies involved salts (e.g. NaCl) mixed
with molecules eliciting different tastes, but a few studies focused on salt mixtures. Thus,
Breslin and Beauchamp (1995) showed that for a mixture of NaCl and KCl, saltiness is
enhanced at low concentration, while at higher concentrations it is suppressed. Lawless et al.
(2003b) studied the taste of CaCl$_2$ in mixture with NaCl and other tastants. These authors
evidenced that saltiness of NaCl was additive with the salty taste of CaCl$_2$ but decrease the
bitter and metallic taste of CaCl$_2$. However, this effect of NaCl on the bitterness of CaCl$_2$ can’t
be generalised to other divalent salts such as MgSO$_4$. Indeed, Breslin and Beauchamp
(1995) and Keast and Breslin (2002a) evidenced little or no suppression of MgSO$_4$ bitterness
by NaCl and sodium gluconate. As suggested in the review made by Keast and Breslin
(2002b), studies on mixture of tastants remained limited. However, this review evidenced that
low intensity/concentration mixtures tend to result in enhancement, medium
intensity/concentration tend to result in additivity, high intensity/concentration tend to result in
suppression.

Thus, due to the concentration levels, the number of ions in mixture and the diversity of
mechanisms putatively involved, phenomena occurring during water consumption were
poorly explored. As a consequence, the objective of the work developed in this first part of
the chapter was to determine the qualitative and quantitative variations in mineral content
occurring in drinking water and to determine whether these variations could have a
perceptual impact on the taste of water.
Chapter II Part 1 – Mineral matrix constituents affecting water taste – Introduction

We first address this question using bottled waters and determined the mineral content range of variation for bottled waters distributed in France. This step allowed us to select bottled waters to be used in a sensory experiment. We evaluated the perceptual impact of total mineral content of water (molarity\(^3\)) but also the nature and proportions of ions included in water, on its taste. The results are reported and discussed in publication 3. In a second step, we determined tap water mineral content variability (publication 4) and verified the impact the observed ionic composition variation on water taste using experimentally designed waters (publication 5).

\[^3\text{In the present manuscript, molarity is the variable used to traduce the total mineral content of water. This refers to the sum of the molarity of different ions dissolved. This definition is generally used for the term osmolarity. However, in the context of taste perception, this word refers to osmotic sensing, that is a specific mechanism. Since other sensory mechanisms could be involved in water taste such as adaptation to saliva, we chose not to use the term osmolarity but molarity.}\]
2. Publication 3:

Beyond molarity, drinking water discrimination is based on ionic pattern variations

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1. Introduction

Drinking water is most often defined as a colourless, tasteless and odourless product. As advocated by Teillet \textit{et al.} (2008), the sensory description of water taste is not an easy task. Nevertheless, despite the difficulty to describe water taste, every one has already experienced taste differences while drinking waters of various origins. For example, some bottled waters have a very specific taste. This taste is due to water mineral content (Burlingame \textit{et al.} 2007). Teillet \textit{et al.} (2009a) demonstrated that the sensory space described by drinking waters relies mainly on their Total Dissolved Solids content. TDS corresponds to the amount of minerals dissolved regardless of their nature and is expressed in mg/L. If TDS content confers to a water its sensory properties, it implies that the taste system can detect osmolarity differences; osmolarity being the solute concentration expressed in mM. This detection could rely either on the dilution of some specific ions contained in saliva which is an adapting solution and constitute a base line for taste (McBurney and Pfaffmann 1963; Matsuo \textit{et al.} 1997), or on hypo-osmotic sensing (Gilbertson \textit{et al.} 2006).

Bruvold (1970) also observed the impact of TDS on water perception. These authors demonstrated an inverse relation between TDS and consumer appraisal. Moreover, these authors demonstrated an impact of qualitative variations of water mineral content, namely the nature of minerals contained in water, on its appraisal. Thus, anions and especially carbonates were found to have a strong negative effect on consumer appraisal (Bruvold and
Gaffey 1969\textsuperscript{a,b}). These results strongly suggest that beyond quantitative variations (i.e. TDS), qualitative variations (i.e. ions nature and proportions) also contribute to water taste. It has been evidenced that the gustatory system is able to detect osmolarity variations but also to detect qualitative differences between ions: Na\textsuperscript{+} is responsible for the typical salty taste whereas Ca\textsuperscript{2+} or Mg\textsuperscript{2+} elicit a bitter-salty taste (Schiffman and Erickson 1971; Lim and Lawless 2005). Most often, these studies relied on the perception elicited by water solutions including a single salt at a suprathreshold concentration. However, natural water is a rather different solution in which ions are present in mixture at peri-threshold concentrations. A few studies were interested in investigating the perception of ions mixtures. Lawless \textit{et al.} (2003b) evidenced that saltiness of NaCl was additive with the salty taste of CaCl\textsubscript{2} but decrease the bitter and metallic taste of CaCl\textsubscript{2}. Keast and Breslin (2002a) did evidence no suppression of MgSO\textsubscript{4} bitterness by NaCl. However, as far as tastants mixture is concerned, the range of concentration could modify the perception of the mixture. Indeed, Breslin and Beauchamp (1995) showed that for a mixture of NaCl and KCl, saltiness is enhanced at low concentration, while at higher concentrations it is suppressed. Studies conducted with mixture at low concentrations tend to evidence enhancement (Keast and Breslin 2002b). Such phenomenon could occur in water which can be considered as a mixture of ions at low concentrations. Stevens (1997; 1998) demonstrated the high level of integration occurring in complex mixtures of compounds at low concentrations. Indeed, this author mixed compounds in proportion of their individual thresholds and measured detection threshold for the resulting mixtures. Results indicated that the concentration of components goes down in the approximate proportion of the number of compounds in the mixture. This integration is valid whatever the compounds to be mixed and whatever their perceptual qualitative similarity. These studies as others (Schifferstein and Kleykers 1996; Uchida and Mainen 2007) performed with tastant or odorant mixtures, suggest that more than the amount of components is the ratios of components is the most important variable. The purpose of the present study was to determine whether the gustatory system is able to discriminate both quantitative and qualitative variations (proportions of ions) of water composition. The main experiment aimed to assess the impact of differences in ionic pattern, molarity and the combined effect of ionic pattern and molarity on discrimination abilities of a panel of untrained subjects. In order to select a pool of water samples to be tested, we first conducted a preliminary study with the objective to select bottled waters with significant ionic pattern differences.
2. Preliminary study: Selection of bottled waters varying on ion proportions

2.1. Materials and methods

2.1.1. Waters

Ionic compositions of 34 bottled waters sold on the French market have been collected by Moisseeff (2008). For each water, we considered the most preponderant ions in terms of concentration (Na\(^+\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), HCO\(_3\)\(^-\), Cl\(^-\), SO\(_4\)\(^{2-}\)). Ion concentration was expressed in mol.L\(^{-1}\) and divided by the water molarity, which is the sum of molarities of the considered ions (Na\(^+\); K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), Cl\(^-\), HCO\(_3\)\(^-\), SO\(_4\)\(^{2-}\)). Thus, we obtained, for each water, the ionic composition expressed in relative proportion of each ion (Table 1).

2.1.2. Data analysis

We performed a Normalized Principal Component Analysis (PCA) using the data reported in Table 1 with the 34 waters as individuals and the 7 ions proportion as variables. This analysis highlighted the most important ions supporting bottled water composition variability. Pearson’s correlation coefficients were determined to analyses relationship occurring between variables. A cluster analysis was carried out in order to identify clusters of water sharing similar ionic patterns. To do so, data were analysed by Hierarchical Cluster Analysis with Euclidean distance and aggregation based on Ward method. PCA and cluster analysis were performed using XL-Stat (Addinsoft, Paris, France).
Table 1: Composition of the 34 bottled waters

<table>
<thead>
<tr>
<th></th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Cl⁻</th>
<th>HCO₃⁻</th>
<th>SO₄²⁻</th>
<th>Molarity</th>
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<td>0.07</td>
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<td>0.05</td>
<td>0.01</td>
<td>0.21</td>
<td>0.33</td>
<td>12.8</td>
</tr>
</tbody>
</table>

Compositions are expressed in proportion of the different ions. Molarity was calculated as the sum of molarities of the different ions (Na⁺; K⁺; Ca²⁺; Mg²⁺; Cl⁻; HCO₃⁻; SO₄²⁻) and is expressed in mM.

2.2. Results

The map corresponding to the PCA analysis was reported on Figure 1. This map shows the most important composition factors describing the waters introduced in this analysis. The two first principal components account for 67% of the total variability. The first principal component accounts for 41% of the total variance and separates waters with a high proportion of Na⁺ and Cl⁻ from the ones including a high proportion of Ca²⁺. Inter-variables correlations, reported in Table 2, confirmed that Na⁺ and Cl⁻ are correlated while both ions were anti-correlated to Ca²⁺. The second component accounts for 26% of the total variability and separates waters with a high proportion of HCO₃⁻ from the ones including a high proportion of SO₄²⁻. Indeed, HCO₃⁻ and SO₄²⁻ variables were found to be negatively correlated (Table 2).
After a hierarchical cluster analysis 4 groups of waters were determined (Figure 2). For the main experiment, one water was selected in each group. Furthermore, within each group, the waters were selected according to the following criteria (i) to have a quite high molarity since it will be diluted in the main study (ii) to be easily available on the market. Therefore, the selected bottled waters were Evian, Taillefine, Carola, and Courmayeur. These selected waters have different ionic patterns (Figure 3) and are representative of the composition variability encountered on the French market.

**Figure 1: PCA of water ionic compositions**

Waters included in the different clusters obtained by hierarchical cluster analysis are represented by different symbols. Names written in italic and solid symbols represent waters selected for the main study.

**Table 2: Inter-variables Pearson correlation matrix**

Values written in bold are significant at p<0.05.
Figure 2: Hierarchical Clustering (Euclidean distance and aggregation based on Ward method) of the 34 waters based on their ionic composition

Figure 3: Ionic pattern of the selected waters
3. Main study: Water samples discrimination

3.1. Materials and methods

3.1.1. Subjects

A number of 115 assessors (65 women and 50 men, ranging from 18 to 68 years old) were recruited on the basis of their availability for this experiment. They signed an informed consent form but the aim of the experiment was not revealed. Participants were asked to avoid smoking, drinking and eating at least one hour before each session and to avoid using perfume the day of the test. They received 10 euros for their participation in a 1-h session.

3.1.2. Water samples

Evian, Taillefine, Carola, and Courmayeur bottled waters, selected in the preliminary experiment for their differentiated ionic composition patterns, were used in this part of the study. Water samples varying on molarity were obtained by dilution of these bottled waters. The 4 bottled waters were diluted with Milli-Q water (water purified and deionised using a Millipore® device). Milli-Q water purity was checked through conductivity and total organic carbon measurements.

The experiment was divided in three blocks in order to test water samples varying on ionic pattern and/or molarity (Figure 4). Each block included different water samples. In the first block, water samples were adjusted at the same molarity with different ionic patterns. To do so, Taillefine, Carola, and Courmayeur bottled water were diluted with Milli-Q water in order to reach the same molarity, which is Evian molarity (9.6 mM). In the second block, 9 water samples were used: Taillefine (original bottled water, 24.8 mM), Taillefine diluted with Milli-Q water and adjusted at 10.2 mM, Taillefine adjusted at 2.05 mM, Carola (bottled water, 20 mM), Carola adjusted at 10.2 mM, Carola adjusted at 2.05 mM, Courmayeur (bottled water, 33.6 mM), Courmayeur adjusted at 10.2 mM and Courmayeur adjusted at 2.05 mM. In the third block, non-diluted waters were used and were the following: Evian (9.6 mM), Taillefine (24.8 mM), Carola (20 mM), and Courmayeur (33.6 mM).

Bottled waters were purchased in 1.5 L plastic bottles from the same lot. Water samples were prepared once for the whole experiment. After preparation, solutions were sterilized using ultraviolet light (SteriPEN® Classic) and stored in 1L glass bottles. Ionic content of each sample was checked after the preparation of the water samples according to AFNOR 2003 for cations (Na⁺, K⁺, Ca²⁺, Mg²⁺), AFNOR 2007a for Cl⁻ and SO₄²⁻ and AFNOR 1996 for HCO₃⁻. Samples stability was checked by pH measurement (Aquatrode Plus with Pt 1000 and 781 pH/Ion meter, Metrohm, Courtaboeuf, France) and conductivity measurement.
(Handled conductivity meter, cond 315i, WTW France S.A.R.L., Ales, France) after preparation and each day of sensory session. All the sensory sessions were conducted within the same week.

### 3.1.3. Sensory procedure

Sensory measurements were performed in a room dedicated to sensory analysis following HACCP and Research Quality Insurance Standards. Consumers were placed in separated booths and their responses were collected using software dedicated to sensory analysis (FIZZ, Biosystèmes, Couternon, France).

Between water samples discrimination capabilities were evaluated using triangular tests (AFNOR 2007b). This discriminative test consisted in the simultaneous presentation of three samples. Two samples were similar, one was different; consumers had to indicate which sample was different.

Subjects’ ability to discriminate water samples varying in ionic pattern, molarity and both was evaluated within a 1-hour sensory session divided into three blocks.

The first block was dedicated to the evaluation of ionic pattern discrimination capabilities. In this block, water samples with different ionic patterns (Evian, Taillefine, Carola, and Courmayeur) but adjusted at the same molarity level (9.6 mM, Figure 4) were compared. Six comparisons were thus performed by each subject: Evian (9.6 mM) vs. Taillefine (9.6 mM), Evian (9.6 mM) vs. Carola (9.6 mM), Evian (9.6 mM) vs. Courmayeur (9.6 mM), Taillefine (9.6 mM) vs. Carola (9.6 mM), Taillefine (9.6 mM) vs. Courmayeur (9.6 mM) and Carola (9.6 mM) vs. Courmayeur (9.6 mM). The second block was dedicated to the evaluation of molarity discrimination capabilities. In this block, water samples varying on molarity but sharing the same ionic pattern were compared. The non-diluted bottled waters and the diluted waters adjusted to 10.2 mM or 2.05 mM, were compared. These comparisons were performed within each of the 3 ionic patterns (Taillefine, Carola, and Courmayeur; Figure 4). As a consequence, 9 comparisons were performed, 3 for each ionic pattern. Combined effect of ionic pattern and molarity on discrimination capabilities was examined in the third block. In this block, non-diluted waters (Evian (9.6 mM), Taillefine (24.8 mM), Carola (20 mM) and Courmayeur (33.6 mM); Figure 4) were compared. Six comparisons were thus performed.

For each triangular test, 10 ml of the three water samples were presented in plastic glasses coded with a three-digit number. Samples presentation plan followed a Williams Latin square. Within each block, triangular tests presentation order also followed a Williams Latin square. The blocks order was also randomised.
### 3.1.4. Data analysis

All statistical analyses were performed using R version 2.9.2. (Copyright © 2009, The R Foundation for Statistical Computing). The data obtained in each block were analysed separately. We performed a first statistical analysis, based on binomial law, with the aim to determine whether water samples were significantly discriminated. Then, we performed a second analysis which aimed at identifying the composition parameters associated with a significant discrimination. This second analysis is based on partial least square regression.

#### 3.1.4.1. Analysis of discriminability

For each triangular test, 2 samples were similar and 1 sample was different. A total of 115 subjects had to decide which sample was different. Correct answers were recorded and allowed to determine correct answer probability (p) occurring in the subjects population. This

---

<table>
<thead>
<tr>
<th>Block 1</th>
<th>Evian (9.6 mM)</th>
<th>Taillefine (9.6 mM)</th>
<th>Carola (9.6 mM)</th>
<th>Courmayeur (9.6 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evian</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Taillefine</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carola</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Courmayeur</td>
<td>1</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Block 2</th>
<th>Non-diluted water</th>
<th>Water diluted at 10 mM</th>
<th>Water diluted at 2 mM</th>
</tr>
</thead>
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<tr>
<td>Non-diluted water</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>Water diluted at 10 mM</td>
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<td>1</td>
<td></td>
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<tr>
<td>Water diluted at 2 mM</td>
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<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Block 3</th>
<th>Evian (9.6 mM)</th>
<th>Taillefine (24.8 mM)</th>
<th>Carola (20 mM)</th>
<th>Courmayeur (33.6 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evian</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
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<tr>
<td>Taillefine</td>
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<td></td>
</tr>
<tr>
<td>Carola</td>
<td></td>
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</tr>
<tr>
<td>Courmayeur</td>
<td>1</td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 4: Comparison between water samples performed within the 3 blocks**

Within Block 2, the non-diluted waters were Taillefine, Carola and Courmayeur.
probability combines the real discrimination probability ($p_d$) and the probability of correct answers occurring by chance ($p_0$). In the case of triangular test, $p_0$ is equal to $\frac{1}{3}$. If $p$ does not differ from $p_0$, water samples should not be considered as different. The correct answer probability ($p$), the 95% confidence interval and the significance of the difference between $p$ and $p_0$ were estimated using the exact binomial test function of R. This procedure allows to determine whether the two water samples compared were perceived as different.

### 3.1.4.2. Analysis of composition parameters

Our hypothesis was that the difference in the proportion of specific ions was at the origin of the perceptual discrimination. Our data analysis strategy relied on the identification of these ions. Since pH depends on water composition, it was not included as a parameter in the preliminary study. However, pH may affect sample discrimination, therefore this parameter has been included as the composition parameter potentially associated with sample discrimination. pH values of the samples is presented Table 3. However, as among the water samples compared, the proportions of some ions are correlated (Table 4), we used the partial least square regression (PLSR; Tenenhaus 1998) to perform this analysis. Indeed, this method is especially adapted when predictor variables are correlated and when there are few samples.

<table>
<thead>
<tr>
<th>Water Sample</th>
<th>pH</th>
</tr>
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<tbody>
<tr>
<td>Carola 2.05</td>
<td>7.91</td>
</tr>
<tr>
<td>Carola 9.6</td>
<td>8.11</td>
</tr>
<tr>
<td>Carola 10.2</td>
<td>8.11</td>
</tr>
<tr>
<td>Carola 20</td>
<td>7.86</td>
</tr>
<tr>
<td>Courmayeur 2.05</td>
<td>7.57</td>
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<td>Courmayeur 9.6</td>
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<td>Courmayeur 33.6</td>
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<tr>
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<td>Taillefine 9.6</td>
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</tr>
<tr>
<td>Taillefine 10.2</td>
<td>7.64</td>
</tr>
<tr>
<td>Taillefine 24.8</td>
<td>7.48</td>
</tr>
<tr>
<td>Evian 9.6</td>
<td>7.46</td>
</tr>
</tbody>
</table>

Table 3: pH of the water samples

Our hypothesis was that the difference in the proportion of specific ions was at the origin of the perceptual discrimination. Our data analysis strategy relied on the identification of these ions. Since pH depends on water composition, it was not included as a parameter in the preliminary study. However, pH may affect sample discrimination, therefore this parameter has been included as the composition parameter potentially associated with sample discrimination. pH values of the samples is presented Table 3. However, as among the water samples compared, the proportions of some ions are correlated (Table 4), we used the partial least square regression (PLSR; Tenenhaus 1998) to perform this analysis. Indeed, this method is especially adapted when predictor variables are correlated and when there are few samples.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Molarity</th>
<th>Ca$^{2+}$</th>
<th>Mg$^{2+}$</th>
<th>K$^+$</th>
<th>Na$^+$</th>
<th>HCO$_3^-$</th>
<th>Cl$^-$</th>
<th>SO$_4^{2-}$</th>
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</thead>
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<tr>
<td>Global</td>
<td>-0.03</td>
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<td>-0.59</td>
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<td>-0.27</td>
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<tr>
<td>K$^+$</td>
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<td>Cl$^-$</td>
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<tr>
<td>SO$_4^{2-}$</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 4: Between variables Pearson correlation matrix

Correlations between variables were calculated on the basis of samples composition differences. Values in bold are significant at $p<0.05$.

PLSR was computed using SIMPLS algorithm with the PLS package described in Mevik and Wehrens (2007).

Individual responses obtained for each triangular tests (0 or 1) were used as dependant variables (Y). Water composition parameters, namely ion proportions and pH, were assigned...
Chapter II Part 1 – Mineral matrix constituents affecting water taste – Publication 3

as independent variables (X). Since the variability associated with judges is important, factor judge was also included in the analysis as independent variable (X). To do so, complete disjunctive table representing judge effect was added to the X table. Thus, cross validation was performed using 115 segments each one corresponding to the responses of a single subject. Jack-knife test was used to determine significance level of the parameter tested regarding discrimination ability (Martens and Martens 2000). The number of axis considered in the study was selected on the basis of root mean squared error of prediction (RMSEP) as function of component numbers.

3.2. Results and discussion

3.2.1. Block 1: ionic pattern discrimination capabilities

In this first block of triangular tests, water samples varying on ionic pattern, but not on molarity (adjusted at 9.6 mM) were compared. Correct answers probabilities (p) obtained from the 6 comparisons performed are presented in Figure 5. The data were first compared to the probability of correct answers occurring by chance (p=1/3). The results indicate that Carola ionic pattern is discriminated from Evian (p=0.0005) and from Courmayeur ionic pattern (p=0.001). Evian ionic pattern also tends to be significantly discriminated from Taillefine ionic pattern (p=0.055). These results demonstrate that the gustatory system is able to discriminate water samples on the basis of ionic pattern only, namely without any variation of molarity.

![Figure 5: Correct answer probabilities associated to comparison of water samples varying on ionic pattern only](image)

All the water samples were adjusted at the same molarity level (9.6 mM). For each of the 6 triangular tests, correct answers probabilities were calculated on the basis of the correct answers obtained from
the 115 subjects. pH differences between the compared water samples are represented by the blue line. The red line represents the correct answers probability occurring by chance (%). Error bars indicated the 95% confidence interval on probabilities. Correct answer probability values and associated significance level are written down above error bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

In order to identify the composition parameters involved in significant discriminations, PLSR was performed on individual subjects’ responses. The results revealed that the 2 first components explained 99.89% of the X variance and 5.65% of the Y variance. This low proportion of Y variance explained is due to low discrimination between samples. Indeed, only 3 comparisons were significant and the proportion of correct answers never exceeded 49%. This means that only 16% of consumers were able to discriminate these samples. Regression coefficients were estimated using Jack-knife test to identify composition parameters associated with sample discrimination (Figure 6). The results indicate that variation of Na\(^+\), Ca\(^{2+}\), pH, Mg\(^{2+}\), HCO\(_3\) are positively associated with sample discrimination. This means that they all could have participated to water samples discrimination. However Na\(^+\) and Ca\(^{2+}\) cations were those varying in the greater proportions; additionally the concentrations of these two ions in the water samples were negatively correlated (table 2). Therefore, one can suggest that these two ions had an important role in samples’ discrimination. Indeed, water samples varying in Na\(^+\) and Ca\(^{2+}\) proportions were especially well discriminated. This is the case for the comparison between Taillefine and Carola which is associated with the highest correct answer probability (p=0.49; discrimination probability being equal to 16%). Taillefine water is characterized by a high proportion of Ca\(^{2+}\), whereas Carola water is associated with a high proportion of Na\(^+\). These findings are reconcilable with data acquired by Schiffman and Erickson (1971) or more recently Lim and Lawless (2005) who demonstrating that Na\(^+\) and Ca\(^{2+}\) or Mg\(^{2+}\) ions elicit different taste perceptions. It is also important to note that pH was positively correlated with sample discrimination. Figure 6 especially evidence the link between pH variation and discrimination. However, all the variables (Na\(^+\), Ca\(^{2+}\), pH, Mg\(^{2+}\), HCO\(_3\)) positively associated with discrimination are correlated. Thus, the comparisons associated with the highest and lowest correct answer probability were also associated with the highest and lowest pH difference. As a consequence, it is difficult to determine among these variables which one is at the origin of the sample discrimination.

On the opposite, variations in Cl\(^-\) or SO\(_4^{2-}\) anions proportions were negatively associated with sample discrimination. This means that water sample varying mainly in Cl\(^-\) and SO\(_4^{2-}\) should have been poorly discriminated. This was actually the case for the comparison between Courmayeur and Taillefine waters, for which the correct answer probability was the lowest (p=0.25). Courmayeur is characterized by a high proportion of SO\(_4^{2-}\) and low proportion of Cl\(^-\), whereas Taillefine is characterized by a high proportion of Cl\(^-\) and low proportion of SO\(_4^{2-}\).
These findings support the idea that the gustatory system has the ability to differentiate ions chemical nature in water, without any variation of the total ions concentration. Nevertheless, it is important to underline that despite their statistical significance, correct discrimination probabilities were low. This suggests that the task was difficult and the perceptual differences between samples very subtle.

![Figure 6: Regression coefficients estimated for comparison of water samples varying on ionic pattern only](image)

Grey bars represent regression coefficients estimated using Jack-knife test performed on the two first PLRS components. Error bars represent standard errors. Regression coefficients and associated significance level are written-down above error bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

These results also suggest that when molarity does not vary, discrimination could be associated either with variations in cation proportions or pH variations. However, the correlation between those variable make difficult to explore further the sensory mechanism putatively involved. In this experiment, cation variations were more associated with discrimination than anion variations. These observations are in line with previously acquired data suggesting that cations could be detected and discriminated by the gustatory system (Lim and Lawless, 2005). Anions were shown to modulate cations perception through a modification of their diffusion into the taste bud along the paracellular way, thus reducing cation access to the receptor or the channel located at the basolateral level (Ye et al. 1991; Lawless et al. 2003a). One can also suggest that the variations in anions concentrations in our samples were not sufficient to alter the perception elicited by cations.
3.2.2. Block 2: molarity difference discrimination capabilities

Within the second block, water samples varying on molarity but sharing the same ionic pattern were compared in order to determine the ability of the gustatory system to differentiate water samples varying on molarity only. Figure 7 presents the correct answers probabilities obtained for the different comparisons performed. All the samples were significantly discriminated. The correct answer probabilities follow molarity differences between the samples indicating that discrimination is linked and even more or less proportional to molarity differences (see green line on Figure 7). It appears that for these samples the discriminability was not associated with pH variations.

![Figure 7: Correct answer probabilities associated to comparison of water samples varying on molarity only](image)

Within this figure comparisons were arranged according to molarity differences between the water samples compared. For each of the 9 triangular tests, correct answers probabilities were calculated on the basis of the correct answers obtained from the 115 subjects. Molarity differences between the compared water samples are represented by the green line whereas pH differences are represented by the blue line. The red line represents the correct answers probability occurring by chance (1/3). Error bars indicated the 95% confidence interval on probabilities. Correct answer probability values and associated significance level are written-down above error bars (** 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

This result is confirmed by PLSR analysis conducted on individual responses of the subjects (Y variable). Absolute values of molarity differences were used as the X variable. Since ionic pattern does not vary between the compared samples in this block, the effect of ionic pattern on the ability to discriminate molarity differences was evaluated by the use of ionic proportions as X variables. Jack-knife test was performed on the two first components which represents 99.89 % of X variance and 1.8 % of Y variance. The results, presented Figure 8,
indicate that molarity variation is the only significant parameter associated with samples
discrimination.

Theses results underline the ability of the gustatory system to perceive variation in total ions
concentration. This is in accordance with the observations of Teillet et al. (2009b). Using
water samples, these authors demonstrated, through a sorting procedure, that the perceptual
space of drinking waters is mainly structured by the TDS amount of the water samples
included in their analysis. TDS is a water composition variable closely related to molarity.
Thus, it seems that the gustatory system has the ability to perceive variations of the total
amount of ion contained in water. Moreover, our results suggest that, since we did not
evidence any impact of the ionic pattern on the more or less good ability to differentiate
waters varying on molarity, one can hypothesized that the mechanism underlying molarity
perception is not specific to ions’ chemical nature.

![Regression coefficients](image)

**Figure 8: Regression coefficients estimated for comparison of water samples varying on
molarity only**

Grey bars represent regression coefficients estimated using Jack-knife test performed on the two first
PLSR components. Error bars represent standard errors. Regression coefficients and associated
significance level are written-down above error bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

### 3.2.3. Block 3: Combined effects of ionic pattern and molarity on
discrimination capabilities

Within the third block, combined effects of ionic pattern and molarity on discrimination
capabilities were examined through the comparison of non-diluted bottled water samples.
The results presented in Figure 9 show that all the comparisons are or tend to be significant.
Correct answer probabilities obtained for the discrimination tests were ordered so as to follow an increasing molarity difference between compared water samples. One can observe that correct answer probabilities do not increase with molarity difference suggesting that when water ionic composition pattern varies, molarity difference is not much involved in sample discrimination. In the same way, it appears that between samples discrimination was not associated with pH variations.

![Correct answer probabilities associated to comparison of water samples varying on both molarity and ionic pattern](image)

For each of the 6 triangular tests, correct answers probabilities were calculated on the basis of the correct answers obtained from the 115 subjects. All the water samples compared were not diluted. Molarity differences between the compared water samples are represented by the green line whereas pH differences are represented by the blue line. The red line represents the correct answers probability occurring by chance (⅓). Error bars indicated the 95% confidence interval on probabilities. Correct answer probability values and associated significance level are written-down above error bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

These observations are confirmed by the results of PLSR analysis. In this analysis, correct answers of the subjects were introduced as Y variable. Absolute values of ionic proportion differences and absolute values of molarity differences between compared samples corresponded to X variables in the analysis. In this case, the three first components were considered and represent 99.9 % of X variance and 7.35% of Y variance. Results indicate that variation in Na⁺, Ca²⁺ proportions between the samples compared were positively associated with discrimination. Indeed, water samples varying on these ions proportion were especially well discriminated (Figure 10). Thus Carola which has a higher proportion of Na⁺ was discriminated from other waters having lower proportion of Na⁺ but higher proportions of Ca²⁺ (the concentration of these two ions in the considered water samples was negatively correlated, see table 4). On the opposite, variations on Cl⁻ proportions were associated with a
lack of discrimination. Indeed, Taillefine water, which has the highest proportion of Cl\(^-\), was discriminated from Evian and Courmayeur waters, which have low Cl\(^-\) proportions, with the lowest probability. It is also important to notice that despite important molarity variations between the compared samples, this last parameter was not found to be linked with water samples discrimination. Since, this parameter was significant when it varied alone which is not the case here, one can hypothesize that qualitative variations have a greater impact and mask the effect of quantitative variations.

**Figure 10: Regression coefficients estimated for comparison of water samples varying on both molarity and ionic pattern**

Grey bars represent regression coefficients estimated using Jack-knife test performed on the three first PLSR components. Error bars represent standard errors. Regression coefficients and associated significance level are written-down above error bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

**4. Conclusion**

In this study, we evaluated the impact of ionic pattern, molarity and their combined effects on water sample discrimination. The results showed that both factors significantly affect discrimination and consequently the taste of water. Results from previous studies (Teillet et al. 2009b) already suggested the significant impact of molarity differences on water sample discrimination. Nevertheless, our results highlighted that when the proportions of ions in water vary to a large extent, these qualitative variations are likely more efficient to induce taste variation as compared to quantitative (molarity) differences. However, due to the correlations between variables it remains difficult, on the basis of the results obtained in this
study, to decipher which composition parameters are involved in water dissolved ion discrimination. To reach this goal, further investigations are needed.
3. Publication 4:

Typology of tap Water in France

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$^3$CIRSEE, Suez Environnement, Le Pecq, France.
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1. Introduction

As reviewed by Petraccia et al. (2006), there are lots of classifications of mineral waters based on mineral content. Some classifications are based on water global mineralization whereas others are based on the predominant chemical elements present in mineral water which have some beneficial effects on health. But none of those classifications are based on water taste. In the same way, most studies conducted on water composition aimed to measure water content for specific minerals and to determine their impact on health (Azoulay et al. 2001; Garzon and Eisenberg 1998; Morr et al. 2006; Rosborg et al. 2006). In these studies, all focused on health, content of different minerals are studied independently and none of them were focused on correlations between mineral amounts. As far as taste perception is concerned, it is important to consider water as a mixture of minerals that are putatively tasty compounds. As a consequence, it is important not to consider independently the different mineral of water. Indeed, several studies demonstrated that odour perception (Uchida and Mainen 2007) but also taste perception are explained (Schifferstein and Kleykers 1996) not only by the amount of individual components but also by ratios of components in the mixture. This is the reason why, the first aim of this study was to identify ionic pattern variations occurring in tap water. That is to say classes of water samples potentially varying on taste.

Another variable, TDS (Total Dissolved Solids) was found to have an important impact on water perception (Teillet et al. 2009b). It corresponds to the quantity of dissolved mineral expressed in mg/L. However, as far as sensory physiology is concerned, this variable is probably not the most accurate one because the minerals which contribute to TDS have different molecular weights. It appears to be more accurate to consider molarities instead of
quantities of the different mineral dissolved in water to relate these data to psychophysical responses. We considered the total molarity of water as the sum of the molarities of the different ions dissolved in water (anions and cations). The second part of this study was to determine the range of variation of tap water molarity for every classes of water we identified in the first part of the study. To do so, we used the database in which Lyonnaise-des-Eaux collects composition analyses performed on the water they distribute.

2. Materials and methods

2.1. Waters

Water suppliers perform frequent analysis on different elements of water composition. Results of the analyses performed by Lyonnaise-des-Eaux on its network are collected in a database. Data analysed in the present study were taken from the 2006 water analyses reports. Water mineral composition was expressed in terms of the following selected ions: Na\(^+\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), HCO\(_3\)\(^-\), Cl\(^-\), SO\(_4\)\(^{2-}\). Ion concentrations (mg/L) were extracted from the analyses reports and transformed into molar concentrations (mol/L). A maximum of two analyses reports were kept for each delivery point. Thus, a total of 1386 analyses reports were used in this study.

2.2. Data analysis

In this study, data analyses were performed using XL-Stat (Addinsoft, Paris, France). Since the objective of the study was to determine ionic pattern variations, compositions were expressed in ion proportion (mol/mol). A cluster analysis was conducted on these ionic proportions to identify clusters of water sharing similar ionic patterns. To do so, data were analysed by Hierarchical Cluster Analysis with Euclidean distance and aggregation based on Ward method. Pearson’s correlation matrix was used to determine correlations occurring between minerals and determine whether some variations in the content of one mineral could be related with variations in another one.

Molarity, that is total dissolved solids express in mol/L is also an important parameter for composition and was studied separately. The water total molarity was calculated using the sum of the molarities of the selected ions and distribution of molarity variations were determined for the whole set of water but also for every class of water we evidenced.

3. Results and Discussion

Since data were collected from a unique water supplier, we decided to study their geographical distribution (Figure 1).
Results indicated that the analysis reports used in the study came from 68 departments (71% of the 96 French departments). All the departments do not represent the same proportions of the analysis performed. This is due both to the presence of Lyonnaise-des-eaux in the department considered and to the importance of the population in this region. Thus, the Paris region which receives a large amount of population is also characterized by a large amount of water analyses.
We first compared the geographical distribution (Figure 1) of the collected data with a French map of the different aquifer types (Figure 2). This comparison indicates that water sampling covered most of different types of aquifer. This suggests that the data analysed in the present study may be considered as representative of the waters distributed at tap in France. Three groups of water could be identified from the cluster analysis (Figure 3). Descriptive analyses were conducted to describe the ionic pattern of these groups (Figure 4). Group 1 represents 61% of the waters included in the analysis. Waters included in this group contain almost exclusively Ca\(^{2+}\) and HCO\(_3^-\). Group 3 represents 18% of the waters analysed and includes waters characterized by balanced proportions of Na\(^+\), Ca\(^{2+}\), HCO\(_3^-\) and Cl\(^-\). Group 2 represents 21% of the data and has an intermediate ionic pattern. In this group, waters have a high proportion of HCO\(_3^-\) as waters included in group 1 and a cationic pattern with more balanced proportions of Na\(^+\) and Ca\(^{2+}\) such as group 3.
Pearson correlations performed on the ionic proportion matrix also account for the relationship between variations of the different ions proportions (Table 1). Proportions of sodium are correlated with proportions of chloride and both are negatively correlated with proportions of calcium and bicarbonates.

<table>
<thead>
<tr>
<th></th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Cl⁻</th>
<th>HCO₃⁻</th>
<th>SO₄²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>0.30</td>
<td>-0.89</td>
<td>0.09</td>
<td>0.72</td>
<td>-0.67</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>K⁺</td>
<td>-0.50</td>
<td>0.23</td>
<td>-0.59</td>
<td>0.61</td>
<td>-0.14</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>-0.52</td>
<td>-0.59</td>
<td>0.61</td>
<td>0.30</td>
<td>0.11</td>
<td>-0.56</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>-0.02</td>
<td>-0.12</td>
<td>0.30</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl⁻</td>
<td>-0.89</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 : Pearson correlation matrix

Values written in bold are significant at p<0.05.
These results have to be compared with results of the clustering performed on bottled water ionic proportions (Chapter 2, Study 1, page - 59 -). The clustering of bottled waters allowed to identify 4 groups of water. The comparison between the ionic pattern of these 4 groups and mean patterns obtained in the actual study indicated that there are less qualitative variations in tap water than in bottled water. Indeed, Evian and Carola groups could be related respectively to group 1 and 3 of tap water clustering. These patterns are especially characterized by 4 ions i.e. Na\(^+\), Ca\(^{2+}\), Cl\(^-\) and HCO\(_3\)\(^-\). Group 2 has a composition varying in between group 1 and 3. None of the two remaining groups identified in the bottled water study exists in tap water. Indeed, Courmayeur bottled water group is characterized by a high content of SO\(_4\)\(^{2-}\) and Taillefine group is characterized by a quite low content of HCO\(_3\)\(^-\) and high content of bivalent cations (Ca\(^{2+}\), Mg\(^{2+}\)). Results obtained in this study indicated that Courmayeur and Taillefine’s patterns do not exist in tap water. K\(^+\) content vary neither in tap water nor in bottled water and remain at a low concentration level. These observations suggested that, as far as tap water and taste are concerned, 4 ions only have to be considered Na\(^+\), Ca\(^{2+}\), Cl\(^-\) and HCO\(_3\)\(^-\).

Molarity variations were also studied and are presented in Figure 5.

![Figure 5: Blox plot of molarity for the 3 groups and for the whole set of water](image)

For each box plot, the bottom and the top of the box are the 25\(^{th}\) and 75\(^{th}\) percentiles. The black line within the box is the median and the red line is the mean. The whiskers extend to the most extreme data point within 1.5x the interquartile range from the box. Any values more extreme than this are marked by a dot.

The mean total molarity for the whole set of water is 8.1 mM. The height of the box plot is not very expended; this means that molarity did not vary a lot across the samples. This is especially true for group 1 which includes the major part of the data. Waters included in group 2 and group 3 presented more variations in molarity. Mean molarity for group 3 is significantly lower than for the other groups (p<0.05). This indicates that waters included in
this group had a lower molarity. It can be observed that a very small proportion of waters had a molarity higher than 13 mM (Figure 5) which is quite low. Even if mean molarity was lower in group 3, some of the water included in this group has high molarity. Thus, for a given molarity level, water samples can have each of the 3 ionic pattern identified.

![Figure 2: Molarity distribution for the whole set of waters](image)

We compared molarity variations observed for tap water with molarity variations observed for bottled water (Chapter 2, Study 1, page - 59 -). This comparison showed that molarity of bottled waters has much higher than the highest values observed for tap water. These narrower variations observed in tap water as compared to bottled water should be due to the fact that most often, bottled waters are selected for their atypical and/or high mineral content. Some of these waters doesn’t even follows standard for drinkable water regarding ionic content (Journal Officiel J.O. n° 122 du 27 mai 2003)

4. Conclusion

To conclude, the present study enables to determine the range of composition variations encountered in tap water. Na\(^+\), Ca\(^{2+}\), HCO\(_3^-\) and Cl\(^-\) were identified to be the most important ions for tap water composition in France. In the same way, it was observed that molarity variations are limited. Molarity mean is 8.1 mM in tap water. Different studies (Teillet et al. 2009a; Chapitre 2, publication 3, page - 59 -) evidenced that bottled water could elicit various tastes. Since our results highlighted that tap water mineral content is less variable than bottled water one, it remains to determine whether tap water composition variations could elicit different tastes.
4. Publication 5:

The Gustatory system discriminates cations at concentrations reconcilable with tap water.

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1. Introduction

Drinking water is considered as a tasteless beverage. However, every one already experienced that some bottled waters elicit a specific taste. Teillet \textit{et al.} (2009b) demonstrated that TDS could account for these perceptual differences. In a previous study, we demonstrated that, beyond molarity, discrimination of drinking waters could also be based on ionic pattern differences. This last study especially suggested that Na\textsuperscript{+} and Ca\textsuperscript{2+} were discriminated. However, it was conducted using diluted bottled waters and variations in the proportions of numerous ions were correlated. As a consequence, it was not possible to identify the ions leading to water discrimination. Additionally, we observed in a previous study (Chapter 2, publication 4, page - 77 -) that the range of variation of tap water molarity is very limited as compared to bottled water.

The aim of the present study was to determine the ability of the gustatory system to discriminate water samples varying on specific ions’ proportions. In order to overcome the limitations encountered in the previous studies, we set up an experimental design which allowed an independent variation of ions’ proportion. Experimental water samples were prepared in order to increase the concentration of a specific ion at 3 different molarity levels (2.05; 6.65 and 10.2 mM) reconcilable with tap water range of molarity.
Chapter II Part 1 – Mineral matrix constituents affecting water taste – Publication 5

2. Materials and methods

2.1. Subjects

The ability of the gustatory system to discriminate between water samples varying only on ionic pattern (i.e. at a constant level of total molarity) was determined using sensory discrimination data obtained from two groups of subjects. The first group of 62 subjects (39 women and 36 men with a mean age of 32±12 years old) performed discrimination tests between water samples at a single molarity level (6.65 mM). The second group of 75 subjects (38 women and 24 men with a mean age of 41±14 years old) performed discrimination tests between water samples at two molarity levels (2.05 mM and 10.2 mM). The ability of the gustatory system to discriminate between water samples varying on the total molarity was performed with a last group of 81 subjects (52 women and 29 men with a mean age of 36±11 years old).

Before recruitment, volunteers were submitted to a short questionnaire in order to discard persons with diseases, medications or health state potentially affecting their sensory sensitivity. The participants were recruited on the basis of their availability for the sensory sessions. They signed an informed consent form but the aim of the experiment was not revealed. Subjects were asked to avoid smoking, drinking and eating at least one hour before each session and using perfume the day of the test. They participated in a unique sensory session lasting one hour. Panellists were paid 10 € for their participation. Within a given group, sensory sessions were conducted within the same week.

2.2. Water samples

Eight ionic patterns were designed to cover a full experimental plan aiming to evaluate the impact of single ion variations on discrimination capabilities.

Experimental water samples were designed to contain a high proportion of only one ion among the following: Na⁺, K⁺, Ca²⁺, Mg²⁺, HCO₃⁻, SO₄²⁻ and Cl⁻ (Table 1 & Figure 1). To do so, a water sample containing a high proportion of a cation (e.g. Na⁺ in water 1, see Table 1) also contained a balanced low amount of other cations (e.g. K⁺, Ca²⁺, Mg²⁺) and an equal amount of anions (HCO₃⁻, SO₄²⁻ and Cl⁻) to ensure electrical neutrality. It is noteworthy that in water patterns with a high proportion of cations, the percentage of the cation added in a larger amount varied depending on its valence. Thus, monovalent cations were adjusted to reach a 40% proportion, whereas divalent cations reached only a 30% proportion, other cations were all adjusted to reach 4% (e.g. K⁺, Ca²⁺, Mg²⁺ in Water 1). In waters including a high proportion of one cation, proportions of anions varied also according to the valence of the cation added in high proportion. When the water had a large proportion of a monovalent ion, the amount of anions was adjusted to ensure electrical neutrality.
cation, anions were adjusted to reach a 16% proportion whereas they reach a 19% proportion in water with a large proportion of a divalent cation. These differences in proportions are due to the necessity to respect electric charge balance. Since water ionic patterns were adjusted to contain the same amount of positive and negative electric charges, water samples did not contain 50% of cations and 50% of anions. The principle was the same for water samples containing a high proportion of a specific anion. A total of 7 water ionic patterns were created on this principle, each one containing a high proportion of one specific ion. Another water ionic pattern (water 8, Figure 1) was designed to be a control. This one had the same proportion of the 4 cations and 3 anions (equilibrated water, Table 1 & Figure 1).

<table>
<thead>
<tr>
<th>Water with a high proportion of</th>
<th>%Na⁺</th>
<th>%Ca²⁺</th>
<th>%Mg²⁺</th>
<th>%K⁺</th>
<th>%HCO₃⁻</th>
<th>%SO₄²⁻</th>
<th>%Cl⁻</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>Water 1</td>
<td>40</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Water 2</td>
<td>4</td>
<td>30</td>
<td>4</td>
<td>4</td>
<td>19</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Water 3</td>
<td>4</td>
<td>4</td>
<td>30</td>
<td>4</td>
<td>19</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>K⁺</td>
<td>Water 4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>40</td>
<td>16</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>Water 5</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>4</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>Water 6</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>4</td>
<td>37</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Water 7</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>Ø</td>
<td>Water 8</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 1: Ion proportions for the water samples

Each line of this table contains the composition of one of the 8 waters designed for the study. Each water is characterized by a high proportion of one ion, except the last water sample which has equivalent proportions of each ion.

Figure 1: Ion proportions for the water samples

Radar charts represent ionic pattern of the water samples. Each sample is characterized by a high proportion of one specific ion (Na⁺, K⁺, Ca²⁺, Mg²⁺, HCO₃⁻, SO₄²⁻ and Cl⁻), except water 8 which is characterized by a balanced ionic pattern with equivalent proportions of each ion.
The 8 ionic patterns were adjusted at 3 different molarity levels: 2.05 mM, 6.65 mM and 10.2 mM. These molarity levels were selected to be reconcilable with Tap water molarity range but also to respect water samples preparation feasibility. For example, it was not possible to produce Water 5 pattern which has a high proportion of HCO$_3^-$ at a molarity higher than 10.2 mM. Beyond this molarity level, this water was not stable and carbonate salts precipitated.

Twenty four water samples (8 ionic patterns x 3 molarity levels) were produced (Table 2).

### Table 2: Salts used for water sample preparation

<table>
<thead>
<tr>
<th>Salts Reference</th>
<th>NaHCO$_3$</th>
<th>Na$_2$SO$_4$</th>
<th>NaCl</th>
<th>Ca(HCO$_3$)$_2$</th>
<th>CaSO$_4$</th>
<th>CaCl$_2$</th>
<th>Mg(HCO$_3$)$_2$</th>
<th>MgSO$_4$</th>
<th>MgCl$_2$</th>
<th>KHCO$_3$</th>
<th>K$_2$SO$_4$</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Hydrogenocarbonate, NORMAPUR VWR Prolabo</td>
<td>0.32</td>
<td>0.25</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Sodium Sulfate, ACS reagent Sigma-Aldrich</td>
<td>0.09</td>
<td>0.11</td>
<td>0.30</td>
<td>0.20</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Sodium chloride, &gt; 99.0% ,ACS reagent Sigma-Aldrich</td>
<td>0.09</td>
<td>0.09</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Calcium carbonate dihydrate 99%, ACS reagent Sigma-Aldrich</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Magnesium carbonate hydroxide hydrate, 99% Sigma-Aldrich</td>
<td>0.09</td>
<td>0.14</td>
<td>0.04</td>
<td>0.20</td>
<td>0.04</td>
<td>0.28</td>
<td>0.04</td>
<td>0.28</td>
<td>0.04</td>
<td>0.28</td>
<td>0.04</td>
<td>0.28</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate, 99.0-102.0%, ACS reagent Sigma-Aldrich</td>
<td>0.09</td>
<td>0.13</td>
<td>0.21</td>
<td>0.09</td>
<td>0.13</td>
<td>0.21</td>
<td>0.09</td>
<td>0.13</td>
<td>0.21</td>
<td>0.09</td>
<td>0.13</td>
<td>0.21</td>
</tr>
<tr>
<td>Magnesium hydrogen carbonate VWR Prolabo</td>
<td>0.24</td>
<td>0.18</td>
<td>0.06</td>
<td>0.18</td>
<td>0.06</td>
<td>0.18</td>
<td>0.06</td>
<td>0.18</td>
<td>0.06</td>
<td>0.18</td>
<td>0.06</td>
<td>0.18</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate, 99.0-102.0%, ACS reagent Sigma-Aldrich</td>
<td>0.09</td>
<td>0.13</td>
<td>0.21</td>
<td>0.09</td>
<td>0.13</td>
<td>0.21</td>
<td>0.09</td>
<td>0.13</td>
<td>0.21</td>
<td>0.09</td>
<td>0.13</td>
<td>0.21</td>
</tr>
<tr>
<td>Magnesium carbonate heptahydrate, 98% Sigma-Aldrich</td>
<td>0.28</td>
<td>0.35</td>
<td>0.99</td>
<td>0.64</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate,98+, ACS reagent Sigma-Aldrich</td>
<td>0.28</td>
<td>0.28</td>
<td>0.15</td>
<td>2.07</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
<td>0.64</td>
<td>0.28</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate,98+, ACS reagent Sigma-Aldrich</td>
<td>0.28</td>
<td>0.28</td>
<td>0.15</td>
<td>2.07</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
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<tr>
<td>Magnesium carbonate heptahydrate, 98%, ACS reagent Sigma-Aldrich</td>
<td>0.28</td>
<td>0.28</td>
<td>0.15</td>
<td>2.07</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
<td>0.64</td>
<td>0.28</td>
</tr>
<tr>
<td>Magnesium carbonate heptahydrate, 98%, ACS reagent Sigma-Aldrich</td>
<td>0.28</td>
<td>0.28</td>
<td>0.15</td>
<td>2.07</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
<td>0.64</td>
<td>0.28</td>
</tr>
<tr>
<td>Magnesium carbonate heptahydrate, 98%, ACS reagent Sigma-Aldrich</td>
<td>0.28</td>
<td>0.28</td>
<td>0.15</td>
<td>2.07</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
<td>0.64</td>
<td>0.28</td>
<td>1.27</td>
<td>0.64</td>
<td>0.28</td>
</tr>
</tbody>
</table>

### Table 3: Concentration of the different salts used for water sample preparation

To avoid off-flavour development due to bacterial growth, solutions were sterilized after preparation using ultraviolet light (SteriPEN® Classic) and stored in 1L glass bottles. Ionic content of the sample was checked after the preparation of the water samples according to AFNOR 2003 for cations (Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$), AFNOR 2007a for Cl$^-$ and SO$_4^{2-}$ and AFNOR 1996 for HCO$_3^-$. The stability of the samples was checked by measurements of pH.
(Aquatrode Plus with Pt 1000 and 781 pH/Ion meter, Metrohm, Courtaboeuf, France) and conductivity (Handed conductivity meter, cond 315i, WTW France S.A.R.L., Ales, France) just after water sample preparation and at the end of the day, after sensory sessions.

Most of water samples was easily prepared through the dissolution of mineral salts (Table 3) in Milli-Q water. Table 4 reported the concentrations of salts corresponding to each water sample preparation recipe.

Ca(HCO$_3$)$_2$ being difficult to dissolve in water, a solution containing this salt was prepared by bubbling CO$_2$ through a solution of Ca(OH)$_2$ prepared with Milli-Q water. Water samples containing this salt were prepared by dissolution of the other salts in this solution (Water 2, 3, 5 and 6; Table 3 & Table 4). Milli-Q water was also added to reach the target molarity level.

**Table 4: Water samples mineral content**

<table>
<thead>
<tr>
<th>Water samples</th>
<th>Na$^+$</th>
<th>Ca$^{2+}$</th>
<th>Mg$^{2+}$</th>
<th>K$^+$</th>
<th>HCO$_3^-$</th>
<th>SO$_4^{2-}$</th>
<th>Cl$^-$</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.05 mM</td>
<td>0.82</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
<td>2.05</td>
</tr>
<tr>
<td>Water 1 (Na$^+$)</td>
<td>0.09</td>
<td>0.61</td>
<td>0.09</td>
<td>0.09</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>2.05</td>
</tr>
<tr>
<td>Water 3 (Mg$^{2+}$)</td>
<td>0.09</td>
<td>0.09</td>
<td>0.61</td>
<td>0.09</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>2.05</td>
</tr>
<tr>
<td>Water 4 (K$^+$)</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.82</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
<td>2.05</td>
</tr>
<tr>
<td>Water 5 (HCO$_3^-$)</td>
<td>0.21</td>
<td>0.21</td>
<td>0.21</td>
<td>0.21</td>
<td>1.02</td>
<td>0.09</td>
<td>0.09</td>
<td>2.05</td>
</tr>
<tr>
<td>Water 6 (SO$_4^{2-}$)</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.76</td>
<td>0.08</td>
<td>0.08</td>
<td>2.05</td>
</tr>
<tr>
<td>Water 7 (Cl$^-$)</td>
<td>0.21</td>
<td>0.21</td>
<td>0.21</td>
<td>0.21</td>
<td>0.09</td>
<td>0.09</td>
<td>1.02</td>
<td>2.05</td>
</tr>
<tr>
<td>Water 8 (Ø)</td>
<td>0.24</td>
<td>0.24</td>
<td>0.24</td>
<td>0.24</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
<td>2.05</td>
</tr>
</tbody>
</table>

| 6.65 mM       | 2.66   | 0.30      | 0.30      | 0.30  | 1.03      | 1.03      | 1.03  | 6.65  |
| Water 1 (Na$^+$) | 0.28   | 1.98      | 0.28      | 0.28  | 1.27      | 1.27      | 1.27  | 6.65  |
| Water 3 (Mg$^{2+}$) | 0.30  | 0.30      | 0.30      | 2.66  | 1.03      | 1.03      | 1.03  | 6.65  |
| Water 4 (K$^+$) | 0.69  | 0.69      | 0.69      | 0.69  | 3.33      | 0.28      | 0.28  | 6.65  |
| Water 5 (HCO$_3^-$) | 0.91  | 0.91      | 0.91      | 0.91  | 2.48      | 0.26      | 0.26  | 6.65  |
| Water 6 (SO$_4^{2-}$) | 0.69  | 0.69      | 0.69      | 0.69  | 0.28      | 0.28      | 3.33  | 6.65  |
| Water 7 (Cl$^-$) | 0.79  | 0.78      | 0.78      | 0.78  | 1.17      | 1.17      | 1.17  | 6.66  |
| Water 8 (Ø) | 4.1    | 0.5       | 0.5       | 0.5   | 1.6       | 1.6       | 1.6   | 10.2  |
| Water 2 (Ca$^{2+}$) | 0.4    | 3.0       | 0.4       | 0.4   | 2.0       | 2.0       | 2.0   | 10.2  |
| Water 3 (Mg$^{2+}$) | 0.4    | 0.4       | 3.0       | 0.4   | 2.0       | 2.0       | 2.0   | 10.2  |
| Water 4 (K$^+$) | 1.1    | 1.1       | 1.1       | 1.1   | 5.1       | 0.4       | 0.4   | 10.2  |
| Water 5 (HCO$_3^-$) | 1.4    | 1.4       | 1.4       | 1.4   | 3.8       | 0.4       | 0.4   | 10.2  |
| Water 6 (SO$_4^{2-}$) | 1.1    | 1.1       | 1.1       | 1.1   | 0.4       | 0.4       | 0.4   | 10.2  |
| Water 7 (Cl$^-$) | 1.2    | 1.2       | 1.2       | 1.2   | 1.8       | 1.8       | 1.8   | 10.2  |
| Water 8 (Ø) | 1.2    | 1.2       | 1.2       | 1.2   | 1.8       | 1.8       | 1.8   | 10.2  |

2.3. Sensory procedure

Sensory measurements were conducted in a room dedicated to sensory analysis following HACCP and Research Quality Insurance Standards. Consumers were placed in separated booths and their responses were collected using a software dedicated to sensory analysis (FIZZ, Biosystèmes, Couternon, France).

Between ionic pattern discrimination capabilities were evaluated using triangular tests (AFNOR 2007b). This discriminative test consisted in the simultaneous presentation of three samples. Two samples were similar, one was different; consumers had to compare the samples and indicate which one was different.
The ability of the gustatory system to discriminate between water samples varying on cations proportion was evaluated through the comparison of water samples including a high proportion of one cation (Water 1(Na$^+$), Water 2(Ca$^{2+}$), Water 3(Mg$^{2+}$), Water 4(K$^+$)). These comparisons were performed at the 3 molarity levels (2.05, 6.65 and 10.2 mM). Similarly, the ability of the gustatory system to discriminate water samples varying on anions proportion was evaluated through the comparison of water samples including a high proportion of one anion (Water 5(HCO$_3^-$), Water 6(SO$_4^{2-}$), Water 7(Cl$^-$)). These comparisons were also performed at the 3 molarity levels (2.05, 6.65 and 10.2 mM).

Comparisons at 6.65 mM were performed by a first group of subjects within a 1-hour sensory session. In this session, triangular test order followed a Williams latin square design. Comparisons at 2.05 and 10.2 mM were performed by a second group of subjects within a 1-hour sensory session. This session was divided into two blocks. The first one was dedicated to comparisons at 2.05 mM and the second one to comparisons at 10.2 mM. Within each block, triangular test order also followed a Williams latin square design.

The capability to discriminate between water samples varying only on molarity was also evaluated. This was done through 3 pairwise comparisons of the equilibrated water samples (Water 8) adjusted at the 3 molarity levels (2.05 mM, 6.65 mM and 10.2 mM). Thus, the 3 comparisons of water samples varying on molarity were performed by a third group of subjects in a dedicated sensory session. As previously, triangular test order followed a Williams latin square design.

Within each triangular test, 10 ml of each three water samples were presented in a plastic glasses coded with a three-digit number; sample orders followed Williams Latin square. Subjects had to wait at least 90 s between each triangular test.

### 2.4. Data analysis

The proportion of correct answers obtained in discrimination tests were used to evaluate the correct answer probability (p). This probability (p) is distributed as a binomial law $[B(n,p)]$ and combines the actual discrimination probability (p$_d$) and the probability of correct answers occurring by chance (p$_0$). In the case of triangular tests, p$_0$ is equal to ⅓. If the correct answer probability (p) does not differ significantly from p$_0$, water samples should not be considered as different. Significance of the difference between p and p$_0$ was determined using the exact binomial test function implemented in R (R version 2.9.2. Copyright © 2009, The R Foundation for Statistical Computing). This function also calculated 95 % confidence intervals associated with correct answer probability (p). This first analysis allows to determine water samples that have been discriminated.

Nevertheless, a second level of analysis was performed in order to determine the factors affecting correct answer probabilities. Several factors were considered such as molarity level.
and of the varying ions proportions. In order to take into account this last factor, the “ion” factor can have two levels: cation or anion, depending on whether the comparison involved water samples containing high proportions of cations or anions. The last factor to be considered is pH difference between water samples. Since water pH is intimately linked with composition, this parameter was not fixed within the experimental plan. Nevertheless, pH differences could account for sample discrimination. Thus, Impact of the aforementioned factors (Molarity, Ion, pH) on discrimination was examined through logistic regression using the GENMOD procedure of SAS (SAS release 9.1.3; SAS Institute Inc., Cary, NC). This procedure fit generalized linear model, on binomial data using logit as link function. Thus, individual answers were used as dependent variable and were coded 0 or 1 depending on whether subject answer was incorrect or correct. Since correct answer probability is distributed as a binomial law \[ B(n,p) \], logit function was used as link function to fit the generalized linear model. Independent variables were subject (factor), molarity level (factor including 3 levels: 2.05mM, 6.65mM and 10.2mM), ion (factor with 2 modalities: anion or cation) and pH (covariate). Post hoc comparisons were performed through Wald chi-square test.

3. Results and Discussion

On the one hand, ability to discriminate between water samples varying on cation and anion proportions was evaluated. This discrimination ability was especially tested at 3 molarity levels. On the other hand, the ability to discriminate water samples varying on molarity only was evaluated through the comparison of water samples including equivalent proportions of ions. Three molarity levels were compared. All these comparisons were performed using triangular tests.

The ability to discriminate between water samples varying on cation and anion proportions was first examined. Correct answer probabilities recorded for the comparisons performed at 2.05 mM are presented Figure 2 and indicated that there is no discrimination between waters at this molarity level. Only one comparison tended to be significant. Thus, water including a high proportion of Ca\(^{2+}\) tended to be discriminated from waters including a high proportion of Mg\(^{2+}\) (p=0.09; Figure 2).
Correct answer probability values and associated significance level are reported on top of the histogram bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

Correct answer probabilities recorded for the comparisons performed at 6.65 mM are presented in Figure 3. At this molarity level, all the waters including a high proportion of cation were discriminated. Thus, water including a high proportion of Na⁺ was discriminated from waters including a high proportion of Mg²⁺ (p=0.002) and K⁺ (p=0.0002) and tended to be discriminated from water including a high proportion of Ca²⁺ (p=0.06; Figure 3). This last water sample including a high proportion of Ca²⁺ was discriminated from waters including a high proportion of Mg²⁺ (p=0.01) and K⁺ (p=0.005; Figure 3). These results suggest that cations are well discriminated even at a low molarity level. On the opposite, waters varying on anionic proportions were not significantly differentiated.
Figure 3: Discrimination of the samples adjusted at 6.65 mM

Each bar of the histogram represents correct answer probability for a comparison between 2 water samples (e.g. the sample including a high proportion of Na$^+$ as compared to the sample including a high proportion of Ca$^{2+}$). pH differences between two compared water samples are represented by the blue line. The red line represents the correct answers probability occurring by chance ($\frac{1}{3}$). Error bars indicated 95% confidence interval. Correct answer probability values and associated significance level are reported on top of the histogram bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

Correct answer probabilities recorded for the comparisons performed at 10.2 mM are presented in Figure 4. At this molarity level, all the water samples including a high proportion of one cation were discriminated or tended to be discriminated from each other (Figure 4). Thus, 5 couples of water were discriminated. The water with a high proportion of Na$^+$ was discriminated from water samples with a high proportion of Ca$^{2+}$ (p=0.001), Mg$^{2+}$ (p=0.04) and K$^+$ (p=0.01). The water including a high proportion of Ca$^{2+}$ was discriminated from the water including a high proportion of Mg$^{2+}$ (p=0.02). In addition, the water including a high proportion of K$^+$ was discriminated from water with a high proportion of Mg$^{2+}$ (p=0.04) and tended to be discriminated from the one with a high proportion of Ca$^{2+}$ (p=0.06). These results confirmed that cations are especially well discriminated.

Only one pair of waters including high proportions of a particular anion (HCO$_3^-$/SO$_4^{2-}$) reached the significant level of discrimination (p=0.04).
Figure 4: Discrimination of the water adjusted at 10.2 mM

Each bar of the histogram represents correct answer probability for a comparison between 2 water samples (e.g. the sample including a high proportion of Na\(^+\) as compared to the sample including a high proportion of Ca\(^{2+}\)). pH differences between two compared water samples are represented by the blue line. The red line represents the correct answers probability occurring by chance (\(\frac{1}{3}\)). Error bars indicated 95% confidence interval. Correct answer probability values and associated significance level are reported on top of the histogram bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

Nevertheless, it could not be excluded that the previous observations could be due to pH differences between the water samples and induced by a variation in ion water composition. The influence of this factor on sensory perception was tested through a logistic regression. This analysis was performed using subject’s individual responses to the discriminative tests as dependant variable. Independent variables were analysed using a sequential order (Type I estimable functions for each effect in the model). The first parameter introduced in the model is the factor “subject” which was significant (p=0.025). Then, pH was introduced in the model as covariate revealing no significant impact of this variable on discrimination (p=0.72). The relationship between pH variation, namely the difference in pH values between two compared water samples, and discrimination abilities is reported on Figure 2, Figure 3 and Figure 4 (blue line). The next tested factor is the molarity level. This factor was found to significantly influence discrimination abilities (p=0.007). Indeed, results showed that discrimination abilities were significantly higher at 10.2 mM than 2.05 mM (\(\chi^2(1,N=1908)=8.13, p=0.004\)). The factor “Ion” (cation or anion) also appeared to significantly influence the discrimination ability (p=0.0008). Waters with a high proportion of one cation were more easily discriminated than water samples with a high proportion of one anion. As
expected, the interaction between the factor molarity and “ion” (cation or anion) tended to be significant (p=0.08). Thus at 2.05 mM, there is no difference between the two modalities of the factor “ion” (cation or anion) ($\chi^2_{(1,N=1908)}=1.1$, p=0.29). Indeed, at this molarity level, none of the comparisons performed was significant. On the opposite, at higher molarity levels, the factor “ion” was significant (at 6.65 mM, $\chi^2_{(1,N=1908)}=12.9$, p=0.0003) or tended to be significant (at 10.2 mM, $\chi^2_{(1,N=1908)}=3.19$, p=0.07). Indeed, at these molarity levels, cations were especially well discriminated as compared to anions.

These observations indicated that, at these concentrations, only cations are detected and discriminated by the gustatory system. This is consistent with general knowledge on the gustatory system. Indeed, several studies suggested that various cations could induce different perceptions. For example, Murphy et al. (1981) demonstrated that KCl elicits a bitter taste whereas NaCl elicits the prototypical salty taste. Thus, discrimination of water sample with a higher proportion of Na$^+$ and K$^+$ may be different. This is actually confirmed by our results, since samples containing a high proportion of Na$^+$ were significantly discriminated from those containing a high proportion of K$^+$ both at 6.65 and 10.2 mM. In a more recent study, Lim and Lawless (2005) performed multidimensional scaling on various salts, demonstrated that CaCl$_2$, MgCl$_2$ and MgSO$_4$ were classified in the group of the bitter compound whereas NaCl was classified in the group of the salty compounds. In this study, the distance between CaCl$_2$ and MgCl$_2$ was higher than the distance between MgCl$_2$ and MgSO$_4$. This suggests that CaCl$_2$ elicit different taste as compared to MgCl$_2$. In our data, the water with a high proportion of Ca$^{2+}$ was discriminated from the water with a high proportion of Mg$^{2+}$ at 6.65 and 10.2 mM.

On the opposite, it has been demonstrated that anions are not directly detected but should modulate cation detection. Indeed, anions could modify the access of the associated cation to the taste receptors located on the basolateral part of the taste cells. This has been observed by Murphy et al. (1981) working on halide salts but also by Lawless et al. (2003a) working on calcium and magnesium salts. These results also helped to understand previous study results (Chapter 2, Publication 3, page - 59 -). This study had for aim to select bottled water with different ionic patterns and to assess their discriminability. We demonstrated that discrimination of drinking water could also be based on ionic pattern differences. Since this study was conducted using diluted bottled waters, variations in the proportions of numerous ions were correlated. As a consequence, it was not possible to clearly identify the composition parameters leading to water discrimination. Our results especially suggested that Na$^+$ and Ca$^{2+}$ could be at the origin of sample discrimination as far as ionic pattern was concerned. pH was also identified as a factor potentially explaining discrimination. Nevertheless, the present study allows to discard pH as an explanatory factor as far as drinking water taste is concerned, namely within the molarity variation range reconcilable.
with tap water. Thus, we can conclude that water ionic patterns are discriminated on the basis of their cationic content.

The second part of the experiment had for aim to evaluate the capabilities of the gustatory system to discriminate between water samples varying on molarity only. This was done by comparison of water samples with equivalent proportions of the different ions, but varying on molarity levels in a range reconcilable with tap water molarity variations. Correct answer probabilities are presented in Figure 5 and indicated that there was no discrimination between waters varying on molarity only.

These results are quite important since they contrast with previous findings. Indeed, in a previous study performed using bottled water (Chapter 2, publication 4, page - 59 -), we demonstrated that waters showing large molarity differences were well discriminated whatever the ionic pattern. In a similar way, Teillet et al. (2009b) used bottled and tap water samples and reported that the taste of water is mainly supported by the total dissolved solids (TDS), a variable highly correlated to molarity. The only explanation which could account for the contrast in these observations is that ionic pattern could also support the taste of water. Indeed, in our previous study as well as in the study conducted by Teillet et al. (2009b), the compared samples were not experimental waters and varied both on molarity level and ionic pattern. On the opposite, in the present study, compared waters were characterised by an atypical ionic pattern with equivalent proportions of the different ions.

![Figure 5: Discrimination of water varying on molarity](image-url)
Each bar of the histogram represents correct answer probability for a comparison between 2 water samples (e.g. the sample at 6.65 mM as compared to the sample at 10.2 mM). pH differences between two compared water samples compared are represented by the blue line. The red line represents the correct answers probability occurring by chance (½). Error bars indicated 95% confidence interval. Correct answer probability values and associated significance level are reported on top of the histogram bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

4. Conclusion

The result of this study demonstrated that discrimination between water samples is associated with difference in their ionic pattern. Differences in cation proportions were especially well detected. The ability to detect these differences increases with the molarity of the samples. On the opposite, water samples varying on molarity only was not significantly discriminated. As a consequence, it appears that the taste of water is supported by both the total mineral content but also on their respective proportions.
5. Partial discussion

Water is often defined as an almost tasteless product. However, early studies demonstrated that water can activate the taste system. Indeed, Zotterman and Diamant (1959) demonstrated that application of water on the tongue of frogs elicited an electrical response of the glossopharyngeal nerve. Studies conducted on drinking water taste rapidly identified Total Dissolved Solids as a factor explaining the water taste perception. Thus, Bruvold (1970) and, Bruvold and Gaffey (1969a,b) evidenced that TDS has an impact on water perception and more specifically on acceptability ratings. More recently, Teillet et al. (2009a) also confirmed this observation using various sensory methodologies.

The investigations conducted on the taste system allow to designate two putative mechanisms that could account for these observations. The first one was investigated by Matsuo et al. (1994) and relies on adaptation to saliva. The second one was investigated by Gilbertson et al. (2006) and relies on the ability of the gustatory system to detect osmotic pressure differences.

Our data may contribute to the understanding of the mechanisms underlying the perception of molarity differences. In the publication 3, results were obtained with more or less diluted bottled waters and showed that the gustatory system is able to detect molarity differences whatever the ionic pattern. This result suggested that only molarity differences may contribute to taste difference between waters. Therefore, the taste of water may only rely on a mechanism that allows osmotic pressure differences to be detected. In contrast, in publication 5, the impact of molarity differences was tested within a more restricted range of molarity, even if included into the range of variation explored within publication 3. Result obtained in publication 5 especially highlighted that subjects were not able to discriminate molarity differences in a restricted range of concentrations. This lack of discrimination was imputed to the specificity of the water ionic pattern of the water used. When comparing the results of both publications, within the same range of molarity (2.05 / 10.2 mM) we can conclude that the detection of molarity differences should depend on the water ionic pattern. Due to this specificity, it seems that these observations more probably rely on an adaptation mechanism as proposed by Matsuo et al. (1994).

Our data also underlined the efficient ability of the gustatory system to discriminate water ionic composition at a very low threshold. Indeed such discrimination was effective as soon as a 8 mM molarity level. We especially established that cations were well discriminated and that discrimination ability increases with concentration (i.e. molarity). These results are in line with data previously reported on the taste system (Murphy et al. 1981; Lim and Lawless 2005) and confirm that both ionic pattern and overall molarity contribute to the taste of water.
Chapter II Part 2 – Cross modal interactions affecting chlorine flavour perception – Introduction

Part 2 – Cross modal interactions affecting chlorine flavour perception

1. Introduction

As stated in the introduction of this second chapter, one of the objectives of the PhD is to explore potential chlorine flavour perceptual neutralisation levers. In the first chapter, we demonstrated that olfaction is the only sensory mechanism activated up to a concentration of 0.3 mg/L, the maximal hypochlorous acid concentration delivered at tap. In the first part of this second chapter, we demonstrated that both ionic pattern and especially cationic pattern but also water total molarity affects the taste of water. Therefore, the approach followed in this chapter relies on the hypothesis that taste induced by water mineral matrix constituents can mitigate chlorine odour perception through smell-taste cross-modal interactions.

Hypochlorous acid (HOCl/ClO\(^{-}\)) pKa is 7.5. Since the pH value for water is usually comprised between 6 and 8, hypochlorous acid is present in its associated form (HOCl, volatile) and dissociated form (ClO\(^{-}\), non-volatile) (Doré 1989). Our findings (Chapter 1, Publication 2, page - 30 -) demonstrated that hypochlorous acid does not activate the gustatory system at concentrations reconcilable with those delivered at tap (<3 mg/L Cl\(_2\)) but likely activates the olfactory system. This suggested that chlorine perception is elicited by the volatile associated form of hypochlorous acid (HOCl). From these results, it is important to notice that a simple modification of water pH, induced by the modification the water mineral matrix, can affect the concentration of hypochlorous acid associated form and consequently chlorine flavour perception. This is consistent with data previously acquired by Bryan et al. (1973) who were the first to measure chlorine perception thresholds. Theses authors demonstrated that the chlorine flavour detection threshold increases when pH decreases that is when the concentration of hypochlorous acid volatile associated form (HOCl) decreases. Thus, it is important to consider the potential impact of mineral matrix on pH and therefore on HOCl concentration and chlorine flavour perception. In such context, it can be expected that water anionic content could be of great importance due to the buffering potential of anions.

Aside physicochemical interactions, sensory interaction could occur between chlorine flavour perception and drinking water taste. Indeed, several studies reported aroma-taste cross-modal interaction.
Chapter II Part 2 – Cross modal interactions affecting chlorine flavour perception –

Introduction

As reviewed by Valentin et al. (2006) and Tournier et al. (2007), many studies focused on odour induced taste enhancement and especially on sweet enhancement induced by aromas. As a basic study, Frank and Byram (1988) evidenced that the sweetness of whipped cream increases when a strawberry aroma is added. The same effect was reported with different aromas and several sweeteners (Cliff and Noble 1990; Clark and Lawless 1994; Prescott 1999). Such cross-modal perceptual interactions have also been demonstrated using other tastes. Thus, Labbe et al. (2006) demonstrated that the addition of cocoa aroma to chocolate beverage can enhance bitterness but does not affect other tastes. Djordjevic et al. (2004) and Lawrence et al. (2009) reported saltiness enhancement induced by various salt-associated odours.

Some authors demonstrated that cross-modal interactions may be modulated by the instructions and the task assigned to the subjects. Indeed, the number of scales used within the sensory procedure can modify apparent interaction strength (Frank et al. 1993; van der Klaauw and Frank 1996). Indeed, the omission of a salient attribute has for consequence dumping effects. Subjects will ‘dump’ their perception of the missing attributes onto the other available scales (Clark and Lawless 1994). This bias can be avoid by the use of multiple and relevant scales (Valentin et al. 2006). If taste-smell interactions have been mostly assessed through intensity ratings, some authors demonstrated significant interactions using unbiased methodologies. Djordjevic et al. (2004) evidenced that sucrose detection was better after sniffing or imagining a strawberry odour has compared to a ham odour. Dalton et al. (2000) demonstrated that panellists had lower benzaldehyde orthonasal olfactory detection threshold when they were tasting in-mouth sub-threshold saccharin solution as compared with water. This effect was not observed when the sweet solution was replaced by an umami solution. These studies also demonstrated that cross-modal interactions are centrally processed and depend on the congruency between taste and smell stimuli. Indeed, Frank et al. (1991) asked to subjects to rate the similarity of all possible combinations of four tastants (sucrose, sodium chloride, citric acid, quinine sulphate) and six odorants (almond, chocolate, lemon, peanut, strawberry, wintergreen). They demonstrated that similarity judgment was a good predictor of the odour induced taste enhancement. On the opposite, it has also been demonstrated that incongruent or novel odours suppress taste intensity (Prescott 1999). If Stevenson et al. (1999) showed that a sweet-smelling odour enhanced the sweetness of sucrose in solution, they also evidenced that it suppressed the sourness of a citric acid solution.

Some authors argued that these odour-taste interactions could be based on learned synesthesia. The acquisition of taste properties by odours can be learned via combined
exposure to the two sensations (Stevenson et al. 1995; Stevenson et al. 1998). In 1995, the authors exposed participants to solutions of relatively unfamiliar aromas (Lychee and water chestnut) in mixture with sucrose or citric acid and clearly demonstrated that lychee and water chestnut odour were rated as significantly sweeter or sourer depending on the taste-smell combination they received during the exposure period.

Mechanisms underlying taste-smell interactions are relatively well understood. Small and Prescott (2005) reviewed the mechanisms occurring during odour/taste integration and the perception of flavour and propose an explanatory model. These authors argued that sensory signals involved in flavour perception are “functionally united when anatomically separated”. Processing of these sensory inputs depends on the prior experience of the particular combination of stimuli, their temporal and spatial occurrence. In their model, neural substrate for this unitary percept are built over time by repeated experience to stimuli. Rolls and Baylis (1994) who identified taste and smell-responsive cells in Orbito-Frontal cortex proposed that bimodal taste/odour neurons developed from unimodal neurons, which originally responded only to olfactory information, through learning of appropriate combinations of signals during repeated co-exposure of particular tastes with odours. These repeated pairings of taste and smell also give rise to their perceptual congruence. However, Small and Prescott (2005) also highlight the asymmetry of this process. Indeed, if odour are likely to acquire taste properties (Stevenson et al. 1995). The opposite relation is not valid. Tastants do not acquired odour properties. Most often taste perceptions are experienced in association with odours whereas odours can be perceived orthonasally and are not systematically associated with tastants. This could explain why many of the studies focusing on taste aroma interactions describe odour induced taste modifications.

Despite this dissymmetry, few studies reported opposite effects, i.e. enhancement or suppression of odour induced by tastants. Bonnans and Noble (1993) studied the impact of various concentrations of citric acid and sucrose on orange-flavoured solutions perceived intensity. They demonstrated that fruitiness intensity and duration was enhanced by both sweetness and sourness, but to a greater extent by sourness. Kuo et al. (1993) studied the impact of sourness, sweetness and saltiness on citral and vanillin odour perception. They demonstrated that citral odour intensity was enhanced by all the tastants. However, the greater enhancement was obtained with acid. On the opposite, vanillin odour intensity was increased by sucrose but decreased by acid or salt.

This short review of the literature on smell-taste interactions underlined that the question of odour suppression by taste has poorly been investigated. Thus, the objective of this chapter is to determine whether drinking water taste supported by minerals can mitigate chlorine
odour perception. Two studies already addressed the question of chlorine flavour interaction with mineral matrix. Piriou et al. (2004) measured chlorine flavour threshold in water varying on TDS and failed to find a significant impact of TDS amount on chlorine flavour detection, whatever subjects' training level. Wiesenthal et al. (2007) also assessed the influence of TDS content on chlorine threshold. These authors measured threshold for chlorine and chloramines in solutions varying on TDS; variation of TDS being produced either by dilution of dechlorinated tap water using miliQ water either using various concentrations of NaCl. To do so, they used Flavour profile analysis which is a standard method for water taste and odour assessment. The comparison of these thresholds suggests that threshold vary according to the composition of the water used and that actual mineral content of water affect chlorine threshold. However, they demonstrated that a synergetic effect of TDS only with one of the two dechlorinated tap water they used. On the opposite, no evidence of any impact of chlorine or chloramine on NaCl threshold was found.

On the bases of these two studies, there is no clear evidence that interactions actually occur between taste, elicited by drinking water mineral content, and chlorine odour perception. However, these observations have to be consolidating and the factor driving these interactions remained to be identified.

In addition, it is important to mention that, during the sensory sessions reported publications 3 and 5 (Chapter 2, pages - 59 -and - 84 -) which had for aim to determine composition parameters affecting drinking water taste, some subjects spontaneously mentioned that some water samples were strongly chlorinated despite they did not contain chlorine. These observations suggest that some minerals could induce a chlorine flavour perception. Therefore, in line with previous results reported in the literature, it could be postulated that perceptual interactions may arise between drinking water taste and chlorine odour. We first address this question using bottled waters in which chlorine was added. We evaluated the impact of total mineral content of water (molarity) but also the nature and proportions of ions included in water (ionic pattern), on the intensity of chlorine odour. Evaluations were performed through paired comparisons. Results are reported and discussed in publication 6.

In a second step, we aimed at exploring further the impact of mineral matrix variations using a different approach based taste and smell intensity ratings of experimentally designed and well-controlled chlorinated water samples. Sensory assessments were performed by a trained panel (Chapter 2, publication 7, page - 116 -).
2. Publication 6:

Tap water chlorine flavour perception increases with water molarity

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1. Introduction

Water chlorination is one of the most common treatments used to ensure an optimal bacteriological safety to tap water. French Public Health Authority imposes a minimal value of 0.3 mg/L Cl₂ at the treatment plant outlet and a minimum of 0.1 mg/L at tap (Journal Officiel 2001). However, chlorine flavour also constitutes one of the major complaints against tap water. Considering data of water composition, tap water appears more chlorinated in some areas of distribution. Water composition and especially mineral contents may be responsible of the taste of water (Chapter 2, Publication 5, page - 84 -). Therefore, it is possible that the variation in chlorine perception at different areas of distribution could be due to physico-chemical and/or sensory interactions between water taste and chlorine flavour. In previous studies (Chapter 1, Publication 2, page - 30 - and Chapter 2, Publication 5, page - 84 -), we demonstrated on the one hand that hypochlorous acid may have the ability to activate the olfactory system at chlorine concentrations reconcilable with those found in tap water. On the other hand, we demonstrated that drinking water can elicit different tastes depending on its molarity and cationic content. The aim of this study is to determine whether modifications of the mineral matrix of water could modulate chlorine flavour perception. Such interactions could be due either to physico-chemical or to sensory interactions.

Bryan et al. (1973) measured chlorine flavour detection thresholds and demonstrated that threshold vary according to the pH of water. This variation was shown to be the results of the pH-driven equilibrium between the non-volatile ionic dissociated form of hypochlorous acid
and its volatile associated form (HOCl). As a consequence, different mineral matrices can confer different pH to water and therefore modify the quantity of hypochlorous acid that may reach and activate the olfactory receptors. Besides, we reported in the bibliographical review that sensory interactions between taste and smell are likely to occur and to modify the aroma and/or taste intensity. As far as chlorine perception in water is concerned, we reported the experiment of Wiesenthal et al. (2007) which demonstrated that NaCl could have an antagonist or a synergic effect on chlorine flavour intensity depending on the ratio of chlorine and NaCl concentrations. On the opposite, Piriou et al. (2004) failed to find a significant impact of TDS on chlorine flavour detection. On the basis of these findings, the purpose of the present study was to explore the role of the water mineral content on chlorine perception for chlorinated waters. We especially wanted to determine whether quantitative and qualitative variations of water composition could modulate chlorine flavour intensity. The impact of differences in ionic pattern, molarity and their combined effect on chlorine flavour intensity has been assessed by a panel of selected consumers using paired comparison tests. Samples to be compared were water samples chlorinated at the same free chlorine concentration but varying in their mineral composition.

2. Materials and methods

2.1. Subjects

A first group of 115 subjects (65 women and 50 men, ranging from 18 to 68 years old) was recruited taking into account their availability during the testing period. The subjects were first submitted to a short questionnaire in order to discard persons with diseases, medications or state potentially affecting their sensitivity. Subjects were then screened to test their ability to discriminate samples varying in chlorine flavour intensity (0.03 vs. 0.3 mg/L Cl\(_2\), 0.01 vs. 0.3 mg/L Cl\(_2\), 0.3 vs. 1 mg/L Cl\(_2\), 0.3 vs. 3 mg/L Cl\(_2\)). These comparisons were performed using waters with different mineral content (i.e. Mont-Roucous, Volvic, Thonon, Vittel, Contrex). During this screening session, subjects performed a total of 20 triangular tests. For each test, subjects were asked to indicate which sample has the highest chlorine intensity. Subjects came to the lab to perform these comparisons within a 1-Hour session. Eighty subjects, who obtained the highest number of correct answers (minimum 12 on 20), were selected to participate to the 1-hour test session. The selection procedure ensured that the group of 80 subjects (44 women and 36 men, ranging from 18 to 68 years old) who finally participated to the test session was able to discriminate chlorine intensity variations in waters eliciting different taste perceptions.

For the two sessions (selection and test), subjects signed an informed consent form. Subjects were informed that the first session had for aim to select people able to discriminate
different chlorine levels. Subjects were asked to avoid smoking, drinking and eating at least one hour before each session and using perfume the day of the test. They were paid 10 € per session for their participation.

2.2. Water samples

In a previous study (Chapter 2, Publication 3, page - 59 -), we evaluated the impact of ionic pattern and/or molarity with modified bottled water (i.e. Evian, Taillefine, Carola, and Courmayeur, Figure 1). In the present study, these 4 bottled waters were diluted with Milli-Q water (water purified and deionised using a Millipore® device) to reach several molarity levels. Milli-Q water purity was checked through conductivity and total organic carbon measurements.

The experiment was divided into three blocks. Each block included different water samples. In the first block, water samples were adjusted at the same molarity. To do so, Taillefine, Carola, and Courmayeur bottled water were diluted with Milli-Q water in order to reach the molarity of Evian (9.6 mM). Thus, within this block, water samples had the same molarity but varied on their ionic pattern composition. In the second block, 9 water samples were used: Taillefine (original bottled water, 24.8mM), Taillefine diluted with Milli-Q water and adjusted at 10.2 mM, Taillefine adjusted at 2.05 mM, Carola (bottled water, 20mM), Carola adjusted at 10.2 mM, Carola adjusted at 2.05 mM, Courmayeur (bottled water, 33.6 mM), Courmayeur...
adjusted at 10.2 mM and Courmayeur adjusted at 2.05 mM. Thus, within this block, water samples varied either on their molarity level, or on their ionic pattern composition. In the third block, non-diluted waters were used: Evian (9.6 mM), Taillefine (24.8 mM), Carola (20 mM), and Courmayeur (33.6 mM).

Bottled waters were purchased in 1.5 L plastic bottles from the same lot. Water samples were prepared once for the whole experiment. After preparation, solutions were sterilized using ultraviolet light (SteriPEN® Classic) and stored in 1L glass bottles. Ionic content of each sample was checked just after the preparation according to AFNOR 2003 for cations (Na⁺, K⁺, Ca²⁺, Mg²⁺), AFNOR 2007a for Cl⁻ and SO₄²⁻ and AFNOR 1996 for HCO₃⁻.

All the water samples were chlorinated by adding sodium hypochlorite (NaOCl ~15%, RECTAPUR, VWR international, France) to reach the same chlorine level, 0.3 mg/L Cl₂. Because of chlorine’s high volatility and degradation by sunlight (UV), water samples were chlorinated the morning of each test day and stored until tasting. Chlorinated samples were thus prepared a maximum of eight hours before tasting and stored in brown glass 500 mL flasks equipped with brown glass stoppers. Flasks and stoppers used were carefully cleaned in order to minimize chlorine demand due to residual compounds which could remain on the glass. The expected chlorine content of the delivered samples was controlled by free and total chlorine measurements. In the absence of chlorine demand, these parameters are assumed to be close to the expected value (0.3 mg/L Cl₂). Both parameters were controlled for each flask just after solution preparation and at the end of each sensory session. These controls were performed through a procedure (pocket colorimeter II, Hach Lange) adapted from the DPD protocol for spectrophotometry (APHA-AWWA-WEF 1998).

Water samples stability was checked by pH measurement (Aquatrode Plus with Pt 1000 and 781 pH/ion meter, Metrohm, Courtaboeuf, France) and conductivity measurement (Handled conductivity meter, cond 315i, WTW France S.A.R.L., Ales, France) after preparation and every day of sensory session. All the sensory sessions were conducted within the same week.

2.3. Sensory procedure

Sensory measurements were performed in a room dedicated to sensory analysis following HACCP and Research Quality Insurance Standards. Consumers were placed in separated booths and their responses were collected using a software dedicated to sensory analysis (FIZZ, Biosystèmes, Couternon, France).

Chlorine flavour intensity of the water samples were compared using paired comparison tests (AFNOR 2007c). This comparison consisted in the simultaneous presentation of two samples. The chlorine content of the samples was the same, samples varied only on their
ionic pattern and/or molarity. Subjects were asked to indicate which one of the two samples had the higher chlorine flavour. This procedure aimed to reveal the interactions (physico-chemical or sensory) occurring between drinking water mineral matrix and chlorine flavour.

All the sensory measurements were performed within a 1-hour sensory session divided into three blocks. In the first block, water samples with different ionic patterns (Evian, Taillefine, Carola, and Courmayeur) but adjusted at the same molarity level (9.6 mM, Figure 2) were compared. Six comparisons were thus performed by each subject: Evian (9.6 mM) vs. Taillefine (9.6 mM), Evian (9.6 mM) vs. Carola (9.6 mM), Evian (9.6 mM) vs. Courmayeur (9.6 mM), Taillefine (9.6 mM) vs. Carola (9.6 mM), Taillefine (9.6 mM) vs. Courmayeur (9.6 mM) and Carola (9.6 mM) vs. Courmayeur (9.6 mM). In the second block, the non-diluted bottled waters and the diluted waters adjusted to 10.2 mM or 2.05 mM, were compared. These comparisons were performed within each of the 3 ionic patterns (Taillefine, Carola, and Courmayeur; Figure 2), and 9 comparisons were performed, 3 for each ionic pattern. In the third block, non-diluted waters (Evian (9.6 mM), Taillefine (24.8 mM), Carola (20 mM) and Courmayeur (33.6 mM); Figure 2) were compared. Six comparisons were thus performed.

For each paired comparison test, 10 ml of the water samples were presented in plastic glasses coded with a three-digit number. Samples presentation plan followed a Williams Latin square. Within each block, the presentation order of the paired comparison tests also followed a Williams Latin square. Within a test session, the blocks’ order was also randomised.
2.4. Data analysis

All statistical analyses were performed using R version 2.9.2. (Copyright © 2009, The R Foundation for Statistical Computing). The data obtained in each blocks were analysed separately. We performed a first statistical analysis, based on binomial law, with the aim to determine whether one water sample was perceived as significantly more chlorinated than the other. Then, we performed a second analysis which aimed to identify the composition parameters associated with significant modulation of the perception of chlorine flavour intensity. This second analysis is based on partial least square regression.

2.4.1. Analysis of the paired comparisons

For each paired comparison test, 2 samples were presented. 80 subjects had to decide which sample elicited the most intense chlorine flavour. For each comparison, the probability

<table>
<thead>
<tr>
<th>Block 1</th>
<th>Evian (9.6 mM)</th>
<th>Taillefine (9.6 mM)</th>
<th>Carola (9.6 mM)</th>
<th>Courmayeur (9.6 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evian (9.6 mM)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Taillefine (9.6 mM)</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Carola (9.6 mM)</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Courmayeur (9.6 mM)</td>
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<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Block 2</th>
<th>Non-diluted water</th>
<th>Water diluted at 10 mM</th>
<th>Water diluted at 2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diluted water</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Water diluted at 10 mM</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Water diluted at 2 mM</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Block 3</th>
<th>Evian (9.6 mM)</th>
<th>Taillefine (24.8 mM)</th>
<th>Carola (20 mM)</th>
<th>Courmayeur (33.6 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evian (9.6 mM)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Taillefine (24.8 mM)</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Carola (20 mM)</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Courmayeur (33.6 mM)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Figure 2: Comparison between water samples performed within the 3 blocks; Within Block 2, the non-diluted water were Taillefine, Carola and Courmayeur.
(p) that a sample was judged as more chlorinated than the other one was compared with probability to designate one of the two samples by chance \(p_0\). This probability \(p_0\) is distributed as a binomial law \[B(n=80, p_0=\frac{1}{2})\].

If \(p\) does not differ from \(p_0\), water samples should not be considered as different on their chlorine flavour intensity. The probability of one sample to be perceived as more chlorinated (\(p\)), the 95% confidence interval and the significance of the difference between \(p\) and \(p_0\) were estimated using the exact binomial test functions of R.

### 2.4.2. Analysis of composition parameters

Our hypothesis was that the difference in the proportion of specific ions or in sample molarity could modulate the perception of chlorine flavour. This modulation induced by the water mineral matrix could generate differences in chlorine flavour intensity while water samples do not vary in their chlorine content. Our data analysis strategy relied on the identification of composition parameters involved in the modulation of chlorine flavour intensity. However, the 4 water samples compared contained 7 different ions, which variations may be correlated. That is the reason why we used the partial least square regression (PLSR; Tenenhaus 1998)) to perform this analysis. Indeed, this method is especially adapted when predictor variables are correlated and when there are few samples. PLSR was computed using SIMPLS algorithm with the PLS package described in Mevik and Wehrens (2007).

For each paired comparison test, one of the two samples was arbitrarily set as the reference. Individual responses obtained for each comparison were coded 0 when the reference was perceived as less chlorinated and 1 when the reference was perceived as more chlorinated. The obtained binary responses were used as dependant variables \(Y\) of the PLSR. Differences in composition parameters (\(\Delta\) ion proportion, \(\Delta\) pH and \(\Delta\) molarity) between the two samples compared were assigned as independent variables \(X\). It is important to underline that the sign of the difference has been conserved. This allow to determine whether the increase of the factor follow the increase of chlorine flavour perception.

In order to take into account variability associated with judges, a complete disjunctive table representing judge effects was added to the \(X\) table. Thus, cross validation was performed using 80 segments each one corresponding to the response of one single subject. Jack-knife test was used to determine significance level of the parameter tested regarding discrimination ability (Martens and Martens 2000). The number of axis considered in the study was selected on the basis of root mean squared error of prediction (RMSEP) as function of component numbers.
3. Results and discussion

3.1. Block 1: Impact of ionic pattern on chlorine flavour intensity

In this first block of paired comparison tests, chlorinated water samples varying on ionic pattern, but not on molarity (adjusted at 9.6 mM), were compared. For each comparison, we determined the probability that one sample (water 1) was perceived as more chlorinated than the other one (water 2). Probabilities (p) obtained from the 6 comparisons performed are presented in Figure 3. These data were first compared with the probability to designate one of the two sample by chance (p=1/2).

![Figure 3: Probability that one of the two water samples was perceived as more chlorinated than the other; water samples vary on ionic pattern only.](image)

All the water samples were adjusted at the same molarity level (9.6 mM) and the same chlorine level (0.3 mg/L Cl\(_2\)). For each of the 6 paired comparisons, probability that water 1 was perceived as more chlorinated than water 2 was calculated on the basis of the answers obtained from the 80 subjects. When p is inferior to 0.5, water 1 was perceived as more chlorinated. When p exceeds 0.5, water 2 was perceived as more chlorinated. The red line represents the probability to designate a sample by chance (½). Error bars indicated the 95% confidence interval on probabilities. Correct answer probability values and associated significance level are written down above error bars ((*) 0.05<p<0.1; * p<0.05; ** p<0.01; *** p<0.001).

The results indicated that chlorinated Carola water was perceived as significantly more chlorinated than chlorinated Taillefine water (p=0.03) and tend to be perceived as more chlorinated than chlorinated Evian water (p=0.06). In order to identify the composition parameters potentially associated with this modulation of chlorine flavour intensity, PLSR was performed on individual responses of the subjects. The results revealed that the first component explained 18.6% of the X variance and 3.4% of the Y variance. It is important to notice that these values are quite low which indicates that this analysis poorly represents...
data variability. This can be explained by the low number of significant comparisons reflecting the difficulty of the sensory task. Nevertheless, regression coefficients were estimated using Jack-knife test and are presented in Figure 4. These results indicate that variation of total chlorine, pH, Mg\(^{2+}\), HCO\(_3^-\) and SO\(_4^{2-}\) are associated with modulation of chlorine flavour perception.

The PLSR coefficient value associated with the pH factor is negative which reveals the inverse relation between pH variation and chlorine flavour perception. This is consistent with data acquired by Bryan et al. (1973) on chlorine flavour perception. Indeed, when pH increases, proportion of hypochlorous acid associated form and chlorine flavour intensity decreases. Similarly, such relation was also observed for total chlorine and SO\(_4^{2-}\), namely, when total chlorine increased or when sulphates’ concentration increased, the chlorine perceived intensity decreased. The relationship between chlorine flavour perception and total chlorine variation underlines that there were slight variations in the chlorine content of water samples. It was difficult to perfectly adjust chlorine content at the same level for all the samples. Nevertheless, the regression coefficient is very low (r=-0.002), which reflects the very low variation in total chlorine content of the samples. Variations of Mg\(^{2+}\) and HCO\(_3^-\) were positively and significantly associated with variations of chlorine flavour perception. This result suggests that waters containing higher concentrations of Mg\(^{2+}\) or HCO\(_3^-\) were those perceived with a higher chlorine flavour intensity. Thus, Evian water which is perceived as more chlorinated than Carola contains a high proportion of HCO\(_3^-\). On the opposite, this relation is less clear for Mg\(^{2+}\), since this ion did not vary a lot between the samples. When taken altogether, these results demonstrate that water ionic pattern could modulate the perception of chlorine flavour. PLSR revealed that pH and composition in anions (HCO\(_3^-\) and SO\(_4^{2-}\)) are the main explanatory variables. This suggests that, at least when total molarity does not vary, the modulation of chlorine flavour intensity induced by water ionic matrix could be mainly driven by physico-chemical mechanisms rather than perceptual mechanisms. Indeed, anions such as sulphates and carbonates are known to have a pH buffering effect. Thus it is likely that the variations in chlorine flavour intensity observed in this experiment were mainly due to pH influencing the concentration of HOCl (hypochlorous acid associated form) available for the olfactory system activation. Moreover, for a perceptual mechanism to be engaged, one could suspect an influence of water taste which could modulate chlorine flavour through cross-modal interactions. However, in a previous study (Chapter 2, publication 3, page - 59 -), we demonstrated that, when molarity does not vary, drinking water taste is based on cationic content. Since cationic content was not found to be an explicative variable in the present study, it is likely that (i) variations of water taste were not
Chapter II Part 2 – Cross modal interactions affection chlorine flavour perception – 
Publication 6
related to variations of chlorine flavour intensity and (ii) cross-modal taste-smell interactions 
does not account for the observed results.

![Image of regression coefficients](image_url)

**Figure 3**: Regression coefficients estimated for comparison of water samples varying on ionic 
pattern only.

Grey bars represent regression coefficients estimated using Jack-knife test performed on the first 
PLRS components. Error bars represent standard errors. Regression coefficients and associated 
significance level are written-down above error bars (***) 0.05<p<0.1; * p<0.05; ** p<0.01; *** p<0.001).

### 3.2. Block 2: Impact of molarity differences on chlorine flavour intensity

Within the second block, chlorinated water samples varying on molarity but sharing the 
same ionic pattern were compared in order to determine the putative impact of molarity 
differences on the perception of chlorine flavour intensity. Figure 4 presents the correct 
answer probabilities obtained for the different comparisons performed. It appears that the two 
comparisons involving the highest molarity differences (Δ molarity between 25-30 mM) 
produced significant results, the sample with the highest molarity being always perceived 
with the highest chlorine flavour (see green line on Figure 4).
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Figure 4: Correct answer probabilities associated to comparison of water samples varying on molarity only

All the water samples were adjusted at the same chlorine level (0.3 mg/L Cl2). For each of the 9 paired comparisons, comparisons are arranged according to the ionic pattern of the water samples compared (4a) or comparisons are arranged according to the molarity difference between the water samples compared (4b).

For each of the 9 paired comparisons, probability that water 1 (W1) was perceived as more chlorinated than water 2 (W2) was calculated on the basis of the answers obtained from the 80 subjects. When p is inferior to 0.5, water 1 is perceived as more chlorinated. When p exceeds 0.5, water 2 is perceived as more chlorinated. Molarity differences between the compared water samples are represented by the green line. The red line represents the correct answers probability occurring by chance (½). Error bars indicated the 95% confidence interval on probabilities. Correct answer probability values and associated significance level are written-down above error bars (*(*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

This result is confirmed by PLSR analysis conducted on individual responses of the subjects (Y variable). Molarity differences were used as the X variable. Since water compared in this block have the same ionic pattern, the effect of ionic pattern on molarity ability to modulate chlorine flavour perception has been assessed using quantity of ion expressed in mmol/L as X variables. Jack-knife test was performed on the two first components which represent 97.9 % of X variance and 1.7 % of Y variance. Indeed, the results of Jack-knife test are presented in Figure 5 and indicate that molarity variation is the only significant parameter associated with sample discrimination. Thus, it seems that chlorine flavour intensity increased as molarity increased. However, it is important to notice that significant chlorine flavour intensity difference occurred for molarity difference higher than 20 mM. This range of variation is high as compared to tap water molarity variation range (Chapter 2, Publication 4, page - 77 -).

Thus, for comparisons in a range of variation reconcilable with tap water (2.05 vs 10.2), no significant differences were observed.
3.3. Block 3: Combined effects of ionic pattern and molarity on chlorine flavour intensity

Within the third block, combined effects of ionic pattern and molarity on chlorine flavour intensity were examined through paired comparisons of non-diluted bottled water samples. The results presented in Figure 6 show that two comparisons were significant and two others tended to be significant. Again, three of these comparisons are those for which the molarity difference was the highest, the sample perceived as the most chlorinated being the one with the highest molarity.
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**Figure 4**: Correct answer probabilities associated to comparison of water samples varying on both molarity and ionic pattern

All the water samples were adjusted at the same chlorine level (0.3 mg/L Cl\(_2\)). For each of the 9 paired comparisons, probability that water 1 was perceived as more chlorinated than water 2 was calculated on the basis of the answers obtained from the 80 subjects. When \( p \) is inferior to 0.5, water 1 is perceived as more chlorinated. When \( p \) exceeds 0.5, water 2 is perceived as more chlorinated. Molarity differences between the compared water samples are represented by the green line. The red line represents the correct answers probability occurring by chance (½). Error bars indicated the 95% confidence interval on probabilities. Correct answer probability values and associated significance level are written-down above error bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

PLSR analysis conducted on individual responses of the subjects (Y variable) also confirmed the effect of water molarity differences. Values of ionic proportion differences and molarity differences between compared samples corresponded to X variables in the analysis. In this case, the first component was considered and represents 96.9 % of X variance and 3.9% of Y variance. Results of Jack-knife test indicate that chlorine flavour intensity increases as molarity increases (Figure 7). On the opposite, results of this test indicate that chlorine flavour intensity increases as HCO\(_3\)^− decreases.

The most significant comparison involved Evian water and Courmayeur. If Courmayeur has the highest molarity, it has also the lowest proportion of HCO\(_3\)^−. This relation is the same for the other significant comparison (i.e. Evian vs. Taillefine or Courmayeur vs. Carola). In such a context, it is difficult to determine if it is the molarity level or the proportion of HCO\(_3\)^− which modulates the perception of chlorine flavour intensity. The comparison between Taillefine and Carola chlorine flavour intensity also tended to be significant. However, this trend is difficult to explain because analysis performed does not evidence a significant factor likely to explain this trend. Indeed, these two waters vary more in their proportions of Na\(^+\) and Cl\(^-\).
Chapter II Part 2 – Cross modal interactions affection chlorine flavour perception –
Publication 6

Figure 7: Regression coefficients estimated for comparison of water samples varying on both molarity and ionic pattern

Grey bars represent regression coefficients estimated using Jack-knife test performed on the three first PLSR components. Error bars represent standard errors. Regression coefficients and associated significance level are written-down above error bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

4. Conclusion

In this study, we evaluated the impact of ionic pattern, molarity and their combined effects on water sample discrimination. The results show that both factors had significant effects on discrimination. Variations in the proportions of anionic content and pH seems to be associated with modulation of the chlorine flavour intensity, possibly due to a mineral matrix-induced modification of the equilibrium between hypochlorous acid associated and dissociated forms. However, it appears that an increase of molarity which increases the taste of water is likely to increase chlorine flavour perception. However, since most variables are correlated, it is important to perform further experiments to determine the mineral matrix composition factors explaining the modulation of chlorine flavour intensity.
3. Publication 7:

**Modulation of chlorine flavour by water mineral matrix: physicochemical or sensory mechanism?**

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1. Introduction

Numerous studies investigated the link between tap water consumption, aesthetic or organoleptic perception and risk perception. Thus, Turgeon et al. (2004) showed that the perception of tap water quality is closely related to the residual chlorine level: people living near a treatment plant who may receive a higher chlorine level in their tap water were generally less satisfied by tap water quality and perceived more risks associated with it than people living far from the plant. Besides, many survey demonstrated that consumers unsatisfied by tap water quality complain about water taste and especially about chlorine taste (Chotard 2008; Suffet et al. 1996). However, very few studies investigated the mechanism underlying drinking water taste and chlorine flavour perception. Knowledge of such mechanism could nonetheless be useful to identify lever likely to reduce chlorine flavour perception in tap water. In previous studies (Chapter 1, publication 2, page - 30 -), we demonstrated that chlorine flavour perception relies on activation of the olfactory system at concentrations reconcilable with those delivered at tap in France. We also demonstrated that total molarity and more particularly cations proportions participate to drinking water taste perception (Chapter 1, publication 3, page - 30 - and publication 5, page - 84 -). Thus, taste-aroma interactions are likely to occur and could affect chlorine flavour perception. In the same way, we demonstrated with chlorinated bottled water (0.3 mg/L Cl₂) varying on composition that both composition parameters and pH may modulate chlorine flavour perception probably via physicochemical interactions (Chapter 2, publication 6, page - 102 -;
Indeed, since hypochlorous acid pKa (pKa=7.54, Doré 1989) fall in the range of water pH, a slight pH variation modifies the content of hypochlorous acid volatile associated form which could account for a modification in chlorine flavour perception. In the same study, we also demonstrated that chlorine flavour intensity increased with molarity. However, these results were obtained with bottled water in a range of molarity higher (25-30mM) than the one observed for tap water (10-15 mM) (Chapter 2, Publication 5, page 84). The molarity of tap waters varies up to 15 mM whereas the molarity of bottled water could reach 30-40 mM. In the same way, four different ionic patterns were identified in bottled water whereas only two were identified for tap water (Chapter 2, Publication 3, page 57). Na\(^+\), Ca\(^{2+}\) Cl\(^-\) and HCO\(^3\)\(^-\) were identified as being the more variable ions in tap water composition.

In such a context, it remains unclear whether cross-modal interactions and/or physicochemical interactions could actually occur between tap water mineral composition and chlorine flavour. That is the reason why, this publication aims to address this question using experimental waters designed to explore the impact of composition variations occurring in tap water on chlorine flavour perception. Most often, sensory interactions are measured owing to scale ratings (Tournier et al. 2009). However, it has been demonstrated that the results obtained from these studies are sensitive to the task assigned to the subject (e.g., (Clark and Lawless 1994). As far as drinking water taste is concerned it seems difficult to assess taste of water using scales. Teillet et al. (2009a) who compared different methodologies to describe drinking water taste demonstrated that methodologies based on sorting task and similarity judgments were more accurate to discriminate waters than multiple scale ratings. Water samples with medium mineral content (e.g. Evian) were described as neutral or tasteless. On the opposite waters with lower or higher mineral content elicited different tastes. These tastes could vary depending on the methodology used. Thus, through sensory profile, Hépar water was described as salty whereas it appeared to be metallic and bitter through temporal dominance of sensation. Waters with low and high mineral content could elicit a large variety of taste (e.g. bitter, metallic, metallic after taste, sour, astringent, dry mouth, ticked throat, salty, etc.) but their descriptions were not always consistent.

These studies point out the difficulty to provide several accurate scales to rate drinking water taste. As a consequence, we chose to use only two scales to assess taste-aroma interactions potentially occurring between chlorine flavour perception and drinking water taste. A global scale was used to assess the taste intensity of drinking water (whatever the taste quality). The second scale aimed to assess chlorine flavour intensity. A preliminary study was first conducted with an internal panel to validate these dedicated psychophysical scales. Then, a panel was recruited and trained to rate water taste and chlorine flavour perception.
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intensity of experimental samples. Chlorine intensity was evaluated via orthonasal and
retronasal route to determine whether modulations of chlorine intensity originated from
physicochemical or sensory interactions. Thus, during the main experiment, subjects
evaluated samples on 3 scales dedicated to measure water taste intensity, chlorine flavour
intensity (via retronasal route) and chlorine odour intensity (via orthonasal route).

2. Preliminary study: Validation of psychophysical scales

2.1. Materials and methods

2.1.1. Subjects

For the preliminary study, a number of 14 assessors (12 women, 2 men), with a mean age of
32±10 years were recruited in the lab and participated to two 1-h sessions. They signed an
informed consent form but the aim of the experiment was not revealed. Participants were
asked to avoid smoking, drinking and eating at least one hour before each session and to
avoid using perfume the day of the test.

2.1.2. Water samples

Seven bottled waters were selected to elicit different taste perception (i.e. Mont-Roucous,
Volvic, Evian, Vittel, Carola, Taillefine, and Courmayeur). These waters, were chosen to
have different ionic pattern and to cover a large molarity range (Figure 1).

![Diagram of water samples with ionic pattern and molarity](image)

**Figure 1: Ionic pattern and molarity of the waters selected for the preliminary study**

Each radar chart represents the ionic content of one water. Composition is expressed in % of ion.
Molarities of waters are written down after their name.
Bottled waters were purchased in 1.5 L plastic bottles from the same lot. All these water samples were chlorinated by adding sodium hypochlorite (NaOCl~15%, RECTAPUR, VWR international, France) to reach 4 chlorine levels: 0 mg/L Cl$_2$, 0.1 mg/L Cl$_2$, 0.3 mg/L Cl$_2$ and 1 mg/L Cl$_2$. Because of chlorine’s high volatility and degradation by sunlight (UV), solutions were daily prepared, stored and monitored according to the procedure describe in Puget, Beno et al. (2010).

Water samples stability was checked by pH measurement (Aquatrode Plus with Pt 1000 and 781 pH/Ion meter, Metrohm, Courtaboeuf, France) and conductivity measurement (Handled conductivity meter, cond 315i, WTW France S.A.R.L., Ales, France) every day of sensory session.

2.1.3. Sensory procedure

Sensory measurements were performed in a room dedicated to sensory analysis following HACCP and Research Quality Insurance Standards. Consumers were placed in separated booths and their responses were collected using a software dedicated to sensory analysis (FIZZ, Biosystèmes, Couternon, France).

Chlorine intensity and water taste intensity were evaluated within two 1-hour sessions. Following a complete experimental design, the 28 samples (7 waters x 4 chlorine levels) were presented in a random order (14 samples per session). For each sample, subjects were asked to rate independently the intensity of two attributes: (i) chlorine flavour and (ii) water taste. Ratings were performed using unstructured linear scales from “No chlorine taste” to “strong chlorine taste” for chlorine intensity and from “No water taste” to “strong water taste” for water taste intensity.

At the beginning of each session, subjects were instructed to evaluate independently chlorine and water taste intensity since samples varied on both parameters. In order to ensure that subjects rated both dimension as independently as possible, they received, at the beginning of the session, two references, one for water taste and one for chlorine intensity and were asked to memorize and rate these reference samples on both scales. These two references were selected on the basis of the result of Teillet et al. (2009b) which demonstrated that water with high and low molarity are likely to elicit tastes whereas waters with medium TDS such as Evian are perceived as neutral. Thus, non-chlorinated Courmayeur water was selected as reference for water taste and Evian chlorinated at 1 mg/L Cl$_2$ was chosen as the reference for chlorine taste. After having evaluated the two references, subjects had to rate the samples and to wait at least 140 s between two samples. They were instructed to rinse their mouth with Evian water during this inter-trial interval.
2.1.4. Data analysis

Statistical analyses were performed using SAS release 9.1.3 (SAS Institute Inc., Cary, NC). Intensity rating data for both attributes were submitted to an ANOVA (Analysis of variance) using the MIXED procedure of SAS. Subjects were considered as a random factor. Correlations of the answers on both scales were also calculated using the CORR procedure of SAS.

2.2. Results and discussion

A first analysis was conducted on water taste and chlorine flavour intensity for non-chlorinated samples.

For water taste, a two-ways ANOVA (judge and water) was conducted and revealed a significant effect of judge ($F_{(13, 98)}=2.01$, $p=0.03$) and water ($F_{(6, 98)}=2.55$, $p=0.026$). These results indicated that the water taste scale allows to discriminate the water samples (Figure 2a).

For chlorine flavour intensity, a two-ways ANOVA was conducted on non-chlorinated samples using the same factors and revealed a significant effect of judge ($F_{(13, 98)}=2.62$, $p=0.004$). Conversely, the factor water ($F_{(6, 98)}=0.64$, $p=0.7$) was not significant. This lack of significance indicates that judges did not confuse chlorine flavour and water taste attributes. Moreover, these results underlines that subjects did not dump sensations elicited by water taste on chlorine flavour scale. This is confirmed by the Pearson correlation coefficient calculated between individual responses obtained for both variables ($r=0.02$, $p=0.85$).

Figure 2: Water taste (2a) and chlorine flavour intensity (2b) rating for non-chlorinated samples

Graph 2a) represents water intensity ratings obtained for non-chlorinated samples. Graph 2b) represents Chlorine flavour intensity ratings for the non chlorinated samples. On both graphs error bars represent 95% confidence interval.
A second analysis was conducted on water taste and chlorine flavour intensity for chlorinated samples in order to determine the impact of chlorine level on both water taste and chlorine flavour intensity (Figure 3).

As far as chlorine flavour intensity is concerned, a two-ways ANOVA revealed a significant effect of judge ($F_{(13, 294)}=2.06, p=0.016$) and chlorine level ($F_{(1, 294)}=27.35, p<0.001$). For water taste intensity, the same analysis was conducted using the same factors and revealed no effect of judge ($F_{(13, 294)}=1.24, p=0.25$) and a weak tendency for chlorine level to be significant ($F_{(1, 294)}=2.84, p=0.09$) suggesting that both scales are not totally independent or rather that chlorine flavour could mask water taste. Indeed dumping cannot account for the evolution of water taste with increasing chlorine level since the intensity of water taste decreased when chlorine concentration increased (see Figure 3).

![Figure 3: Water taste (3a) and chlorine flavour intensity (3b) rating according to chlorine level for all the samples](image)

Results of this preliminary study indicated that, at least for non-chlorinated samples, subjects do not confuse water taste and chlorine flavour intensity meaning that those scales are valid to measure putative interactions between water taste and chlorine flavour perception.
3. Main study: Modulation of chlorine flavour by water mineral matrix

3.1. Materials and methods

3.1.1. Subjects and training

40 volunteers, available for the duration of the study, were invited to participate in a selection session. The selection tests consisted in the European test of olfactory capabilities (ETOC, Thomas-Danguin et al. 2003), a mental concentration test (Bourdon Test, Lesschaeve 1997), a test to evaluate subjects ability to rank six chlorine solutions through retronasal and orthonasal routes and 3 triangular tests aiming to evaluate their ability to discriminate water taste. 14 out of 40 subjects were selected on the basis of their results to these tests. Selected subjects participated to 8 1-hour sessions of training. These sessions aimed to train panellists to rate independently water taste and chlorine flavour intensity. As far as water taste intensity is concerned, panellists were first familiarized with different water tastes. To do so, subjects were asked to sort water samples according to similarity. Training went on with a ranking task in which subjects had to order water samples according to water taste intensity. Then, panellists were trained to rate water taste intensity for various samples including bottled and experimental waters. For the training on water taste, panellists were informed that the samples they tasted were not chlorinated.

Subjects were also trained to rate chlorine odour and flavour intensity respectively through orthonasal and retronasal evaluations. For chlorine flavour intensity, subjects were instructed not to smell the samples before tasting and were informed they had to evaluate the same water chlorinated at different chlorine levels. Training on chlorine scales consisted in ranking and rating exercises. During those exercises, panellists were informed that chlorinated samples were always prepared using the same water. The last training session was dedicated to rate water samples varying on taste and chlorine intensity using the 3 scales for which they were trained (water taste, chlorine flavour and chlorine odour).

3.1.2. Water samples

Na⁺, Ca²⁺, Cl⁻ and HCO₃⁻ were identified in a previous study (Chapter 2, publication 5, page - 84 -) as being the ions which vary the most in tap water composition. Therefore, 8 ionic patterns were designed to evaluate their impact both on water taste and chlorine flavour perception (Figure 2). However, since drinking water contains also K⁺, Mg²⁺, SO₄²⁻, these ions were also included to the formulation of the water samples.
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![Image of ionic patterns and molarity of experimental waters](image)

**Figure 4: Ionic pattern and molarity of the experimental waters designed for the main experiment**

Each radar chart represents the ionic content of one water. Composition is expressed in % of ion. Water 1 to 4 had high proportion of a specific ion whereas water 5 to 8 had a high proportion of a specific cation and a specific anion. The 8 waters were adjusted at two molarity levels: 2.05 mM and 10.2 mM.

Four water samples were designed to contain a high proportion of Na⁺, Ca²⁺, Cl⁻ or HCO₃⁻. Thus, a water sample with a high proportion of a specific cation (e.g. Na⁺ in water 1, see Figure 4 & Table 1) also contained a low and balanced amount of other cations (e.g. K⁺, Ca²⁺, Mg²⁺ in water 1) and an equal amount of anions (HCO₃⁻, SO₄²⁻ and Cl⁻ in water 1) to ensure electrical neutrality. It is the reverse for water with a high proportion of a specific anion. Four water samples were also designed to contain a high proportion of a specific cation and a specific anion (i.e. Na⁺ and Cl⁻, Na⁺ and HCO₃⁻, Ca²⁺ and Cl⁻, Ca²⁺ and HCO₃⁻; see Figure 4 & Table 1). Such waters also contained a low and balanced amount of other cations and anions (Figure 4 & Table 1, water 5 to 8).

It is noteworthy that the percentage of cation or anion added in a larger amount varied depending on their valence. These differences in proportions are due to the necessity to respect electric charge balance. Since water ionic patterns were adjusted to contain the same amount of positive and negative electric charges, water samples did not contain 50% of cations and 50% of anions. The principle was the same for water samples containing a high proportion of a specific anion.
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<table>
<thead>
<tr>
<th>Water with a high proportion of</th>
<th>%Na⁺</th>
<th>%Ca²⁺</th>
<th>%K⁺</th>
<th>%Mg²⁺</th>
<th>%Cl⁻</th>
<th>%HCO₃⁻</th>
<th>%SO₄²⁻</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>40</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2</td>
<td>29</td>
<td>4</td>
<td>4</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>3</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>40</td>
<td>8</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>3</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>8</td>
<td>40</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>Na⁺ &amp; Cl⁻</td>
<td>40</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>40</td>
<td>5</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Na⁺ &amp; HCO₃⁻</td>
<td>40</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>40</td>
<td>5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺ &amp; Cl⁻</td>
<td>3</td>
<td>30</td>
<td>3</td>
<td>3</td>
<td>40</td>
<td>11</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>Ca²⁺ &amp; HCO₃⁻</td>
<td>3</td>
<td>30</td>
<td>3</td>
<td>11</td>
<td>40</td>
<td>11</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Ion proportions for the water samples

Each line of this table contains the composition of one of the 8 water designed for the study. Water 1 to 4 are characterized by a high proportion of one ion whereas water 5 to 8 are characterized by a high proportion of one cation and one anion.

The 8 ionic patterns were adjusted at 2 different molarity levels: 2.05 mM, and 10.2 mM. These molarity levels were selected to be reconcilable with tap water molarity range but also to respect water samples preparation feasibility. For example, it was not possible to produce Water 4 pattern which has a high proportion of HCO₃⁻ at a molarity higher than 10.2 mM. Beyond this molarity level, this water was not stable and bicarbonate salts precipitated.

Sixteen water samples (8 ionic patterns x 2 molarity levels) were produced (Table 2).

<table>
<thead>
<tr>
<th>Water samples</th>
<th>Mineral content expressed in mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺</td>
</tr>
<tr>
<td>Water 1 (Na⁺)</td>
<td>0.82</td>
</tr>
<tr>
<td>Water 2 (Ca²⁺)</td>
<td>0.09</td>
</tr>
<tr>
<td>Water 6 (Na⁺ &amp; HCO₃⁻)</td>
<td>0.06</td>
</tr>
<tr>
<td>Water 7 (Ca²⁺ &amp; Cl⁻)</td>
<td>0.05</td>
</tr>
<tr>
<td>Water 1 (Na⁺)</td>
<td>4.10</td>
</tr>
<tr>
<td>Water 2 (Ca²⁺)</td>
<td>0.44</td>
</tr>
<tr>
<td>Water 3 (HCO₃⁻)</td>
<td>1.07</td>
</tr>
<tr>
<td>Water 4 (Cl⁻)</td>
<td>1.11</td>
</tr>
<tr>
<td>Water 5 (Na⁺ &amp; Cl⁻)</td>
<td>4.10</td>
</tr>
<tr>
<td>Water 6 (Na⁺ &amp; HCO₃⁻)</td>
<td>4.10</td>
</tr>
<tr>
<td>Water 8 (Ca²⁺ &amp; HCO₃⁻)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table 2: Mineral content of the water expressed in mM
NaHCO₃ Sodium Hydrogenocarbonate, NORMAPUR VWR Prolabo 27778.236
Na₂SO₄ Sodium Sulfate, ACS reagent Sigma-Aldrich 238597
NaCl Sodium chloride, > 99.0%, ACS reagent Sigma-Aldrich S9888
Ca(HCO₃)₂ This solution was produced using Ca(OH)₂ and CO₂
Ca(OH)₂ Akdolit H, > 92% Ca(OH)₂ Chaux de Boran Einecs 2151373
CO₂ ALPHAGAZ™ CO₂ SFC Air Liquide 1066
CaSO₄ Calcium sulfate dihydrate 98%, ACS reagent Sigma-Aldrich 255548
CaCl₂ Calcium chloride dihydrate 98+%, ACS reagent Sigma-Aldrich 223506
Mg(HCO₃)₂ Magnesium sulfate heptahydrate, 98%, ACS reagent Sigma-Aldrich 227668
MgSO₄ Magnesium carbonate hydroxide hydrate, 99% Sigma-Aldrich 230391
MgCl₂ Magnesium chloride hexahydrate, 99.0-102.0%, ACS reagent Sigma-Aldrich M9272
KHCO₃ Potassium hydrogen carbonate VWR Prolabo 102064H
K₂SO₄ Potassium sulfate, ACS reagent Merck 105153
KCl Potassium chloride, NORMAPUR VWR Prolabo 26764.232

Table 3: Salts used for water sample preparation

<table>
<thead>
<tr>
<th>Water samples</th>
<th>NaHCO₃</th>
<th>Na₂SO₄</th>
<th>NaCl</th>
<th>Ca(HCO₃)₂</th>
<th>CaSO₄</th>
<th>CaCl₂</th>
<th>MgSO₄</th>
<th>MgCl₂</th>
<th>KHCO₃</th>
<th>K₂SO₄</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water 1 (Na⁺)</td>
<td>0.32</td>
<td>0.03</td>
<td>0.05</td>
<td>0.20</td>
<td>0.21</td>
<td>0.20</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Water 2 (Ca²⁺)</td>
<td>0.17</td>
<td>0.05</td>
<td>0.05</td>
<td>0.22</td>
<td>0.17</td>
<td>0.05</td>
<td>0.05</td>
<td>0.22</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Water 3 (Cl⁻)</td>
<td>0.22</td>
<td>0.19</td>
<td>0.03</td>
<td>0.03</td>
<td>0.17</td>
<td>0.05</td>
<td>0.22</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Water 4 (HCO₃⁻)</td>
<td>0.82</td>
<td>0.02</td>
<td>0.04</td>
<td>0.01</td>
<td>0.05</td>
<td>0.03</td>
<td>0.03</td>
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<td>0.03</td>
</tr>
<tr>
<td>Water 5 (Na⁺ &amp; Cl⁻)</td>
<td>0.06</td>
<td>0.11</td>
<td>0.10</td>
<td>0.41</td>
<td>0.13</td>
<td>0.11</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Water 6 (Na⁺ &amp; HCO₃⁻)</td>
<td>0.06</td>
<td>0.35</td>
<td>0.16</td>
<td>0.11</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table 4: Concentration of the different salts used for water sample preparation

Most part of water samples was easily prepared through the dissolution of mineral salts (Table 3) in Milli-Q water. Table 4 reported the concentrations of salts corresponding to each water sample preparation recipe.

Ca(HCO₃)₂ being difficult to dissolve in water, a solution containing this salt was prepared by the bubbling of CO₂ though a solution of Ca(OH)₂ prepared with Milli-Q water. Water samples containing this salt were prepared by dissolution of the other salts in this solution (Water 2, 4, 5, 7 and 8; Table 3 & Table 4). Milli-Q water was also added to reach the target molarity level. Ionic content of the sample was checked after the preparation of the water samples according to AFNOR 2003 for cations (Na⁺, K⁺, Ca²⁺, Mg²⁺), AFNOR 2007a for Cl⁻ and SO₄²⁻ and AFNOR 1996 for HCO₃⁻. To avoid off-flavour development due to bacterial growth, solutions were sterilized after preparation using ultraviolet light (SteriPEN® Classic) and stored in 1L glass bottles.
For retronasal evaluations, a total of 32 samples were delivered to the panellists: the 8 ionic patterns adjusted at two molarity levels (2.05 Mm and 10.2 mM) but also chlorinated at two chlorine levels, 0 and 0.3 mg/L Cl₂.

For orthonasal evaluations, the 16 chlorinated samples were delivered (8 ionic patterns x 2 molarity levels adjusted at 0.3 mg/L Cl₂).

Water samples were chlorinated by adding sodium hypochlorite (NaOCl~15%, RECTAPUR, VWR international, France) to reach the same chlorine level, 0.3 mg/L Cl₂. As for the preliminary study, solutions were daily prepared and stored according to the procedure describe in Puget, Beno et al. (2010). The expected chlorine content of the delivered samples was daily controlled through a procedure (pocket colorimeter II, Hach Lange) adapted from the DPD protocol for spectrophotometry (APHA et al., 1998). These controls were performed just after solution preparation and at the end of each sensory session. Water samples stability was also checked by pH measurement (Aquatrode Plus with Pt 1000 and 781 pH/Ion meter, Metrohm, Courtaboef, France) and conductivity measurement (Handled conductivity meter, cond 315i, WTW France S.A.R.L., Ales, France) after preparation and every day of sensory session.

3.1.3. Sensory procedure

As for the preliminary study, sensory measurements were performed in a room dedicated to sensory analysis and panellists' responses were collected using a software dedicated to sensory analysis (FIZZ, Biosystèmes, Couternon, France).

Panellists participated in three 1-hour sessions which constitutes 3 repetitions of the evaluations. Within each session, panellists had first to rate chlorine odour intensity of the 16 chlorinated water samples (8 ionic patterns * 2 molarity levels). For these orthonasal evaluations, chlorinated samples were presented in 60 ml brown glass flask containing 20 ml of samples. The presentation order was different in each sensory session and followed a Williams Latin Square. The second part of the 3 sensory sessions was dedicated to the retronasal evaluation of the water samples. Panellist had to rate water taste and chlorine flavour intensity for 32 water samples (8 ionic patterns * 2 molarity levels * 2 chlorine level (0 and 0.3 mg/L Cl₂)). To do so, they received 10mL of water samples delivered in plastic glasses equipped with a cap. As for the training, panellists were instructed not to smell the samples before retronasal evaluations. They had to wait 1 min between two samples and to rinse their mouth with Evian water. Sample order followed a Williams Latin Square and was different in each of the 3 sessions.
3.1.4. Data analysis

Data analyses were carried out with SAS release 9.1.3 (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was performed with mixed linear models using the MIXED procedure of SAS with panellists and repetitions as random factors. Type 1 error mean square analysis was conducted meaning that factors are tested in the order they appear in the model. Post-hoc comparison of LS (Least Square) means was performed using Bonferroni adjustment for multiple comparisons. To analyze correlations between variables, Pearson’s correlation coefficients were calculated using the CORR procedure of SAS.

3.2. Results and discussion

3.2.1. Orthonasal evaluations: Chlorine odour

An ANOVA model including all parameters was first estimated to explain chlorine odour intensity ratings. As presented in Table 5, pH variations tend to affect chlorine odour intensity ($F_{1, 619}=3.60$, $p=0.06$) and this effect varies according to molarity level ($F_{1, 619}=6.52$, $p=0.01$). Ionic pattern also influenced significantly chlorine odour intensity ($F_{7, 619}=3.03$, $p=0.004$) and this effect depends on molarity level ($F_{7, 619}=2.42$, $p=0.02$).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>1</td>
<td>619</td>
<td>3.60</td>
<td>0.06</td>
</tr>
<tr>
<td>Free chlorine</td>
<td>1</td>
<td>619</td>
<td>0.06</td>
<td>0.81</td>
</tr>
<tr>
<td>Total chlorine</td>
<td>1</td>
<td>619</td>
<td>1.31</td>
<td>0.25</td>
</tr>
<tr>
<td>Molarity</td>
<td>1</td>
<td>619</td>
<td>0.04</td>
<td>0.84</td>
</tr>
<tr>
<td>pH*Molarity</td>
<td>1</td>
<td>619</td>
<td>6.52</td>
<td>0.01</td>
</tr>
<tr>
<td>Free chlorine * Molarity</td>
<td>1</td>
<td>619</td>
<td>1.76</td>
<td>0.19</td>
</tr>
<tr>
<td>Free chlorine * Molarity</td>
<td>1</td>
<td>619</td>
<td>2.03</td>
<td>0.15</td>
</tr>
<tr>
<td>Ionic Pattern</td>
<td>7</td>
<td>619</td>
<td>3.03</td>
<td>0.004</td>
</tr>
<tr>
<td>Molarity * Ionic Pattern</td>
<td>7</td>
<td>619</td>
<td>2.42</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 5: Results of analysis of variance on chlorine odour intensity performed with a mixed linear model: Test of fixed effects using type 1 error mean square

Since several factors related to water mineral matrix were found to affect chlorine odour intensity it is obvious that physicochemical interactions occur. Indeed, in the orthonasal condition, subjects did not put the samples in mouth but only smell above the cup. As a consequence, differences in chlorine odour intensity could only be induced by a modification of HOCl concentration which is likely the result of the physicochemical influence of the water mineral matrix on HOCl release in the headspace. Since pH and Ionic pattern effects depend on molarity, two models (one for each molarity level) were estimated to investigate the relations between chlorine odour and independent variables. At the low molarity level, the model computation indicates that pH is the only significant factor influencing chlorine odour ($F_{1, 302}=5.57$, $p=0.02$; Table 6).
Table 6: Results of analysis of variance on chlorine odour intensity performed with a mixed linear model at low and high molarity: Test of fixed effects using type 1 error mean square

Chlorine odour intensity increases as pH decreases. As the olfactory system is activated by hypochlorous acid associated form (HOCl) which is volatile, we conclude that HOCl concentration should increase as pH decreases (Figure 5). These results are in line with knowledge on hypochlorous acid chemistry. Indeed this acid is a weak acid with a pKa value of 7.54 (Doré 1989). As reported on Figure 5, pH values of all samples fall above this pKa value. As a consequence, when pH decreases from 8.2 to 7.5, the equilibrium between the dissociated form of hypochlorous acid (ClO\(^-\)) and its associated form (HOCl) is displaced in favour of the associated form. This means that the lower the pH the higher the HOCl volatile form concentration and the higher the concentration in the headspace. In conclusion, when pH decreases, the concentration of HOCl entering into the nose increases and the chlorine odour is more intense.
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Figure 5: Mean chlorine odour intensity rating and pH value for water samples adjusted at 2.05 mM

Black bars represent chlorine odour intensity mean and are estimated through the LSMEANS instruction of the MIXED procedure of SAS. Error bars are 95% confidence interval. The blue line represents the pH value for each sample.

At high molarity level, the ANOVA revealed that the ionic pattern is the only significant factor (F(1, 302)=3.99, p=0.0003; Table 6). Figure 6 shows chlorine odour intensity mean obtained for the different ionic patterns. The water with a high proportion of Na\(^+\) and HCO\(_3\)- was perceived with the lowest chlorine odour intensity whereas the water sample containing a high proportion of Cl\(^-\) had the highest chlorine odour intensity. Such impact of mineral composition can only be explained by physicochemical interaction leading a different HOCl amount in the headspace above the water samples.

Figure 6: Mean chlorine odour intensity rating for water samples adjusted at 10.2 mM

Black bars represent chlorine odour intensity mean and are estimated through LSMEAN option of the mixed procedure. Error bars are 95% confidence interval. Samples with different letters are significantly discriminated (p<0.05).

The observed differences in odour intensity cannot be attributed to variations in sample chlorination since free and total chlorine are not significant factors in the ANOVA. If it is known that ions may have various buffering power, ANOVA results did not evidence a clear role of pH. Therefore, the chemical mechanisms that could account for these findings remain unclear and would need further physicochemical investigations.
3.2.2. Retronasal evaluations: water taste and chlorine flavour

3.2.2.1. Water taste intensity

An ANOVA model including all parameters was first estimated to explain water taste intensity ratings. Results presented in Table 7 evidence a significant effect of ionic pattern ($F_{(1,1270)}=8.1$, $p<0.0001$) and molarity ($F_{(1,1270)}=64.8$, $p<0.0001$).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>1</td>
<td>1270</td>
<td>0.5</td>
<td>0.50</td>
</tr>
<tr>
<td>Chlorine level</td>
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<td>1270</td>
<td>2.0</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Molarity</strong></td>
<td>1</td>
<td>1270</td>
<td>64.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Molarity * pH</td>
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<td>1270</td>
<td>0.1</td>
<td>0.73</td>
</tr>
<tr>
<td>Molarity * Chlorine level</td>
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<td>1270</td>
<td>1.6</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Ionic pattern</strong></td>
<td>7</td>
<td>1270</td>
<td>8.1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Ionic pattern * Molarity</td>
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<td>1270</td>
<td>1.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Ionic pattern * Chlorine level</td>
<td>7</td>
<td>1270</td>
<td>0.7</td>
<td>0.651</td>
</tr>
</tbody>
</table>

Table 7: Results of analysis of variance on water taste intensity performed with a mixed linear model: Test of fixed effects using type 1 error mean square

Water with a high molarity elicits a lower taste ($M=3.5$, $SD=0.7$) than water samples with a lower molarity ($M=4.8$, $SD=0.7$) ($p<0.001$, Figure 7a). Figure 7b presents water taste ratings for the different ionic patterns and evidenced that the ionic patterns containing a high proportion of $Na^+$ elicited a higher taste than the other samples. These results are consistent with results acquired in previous studies showing that discrimination of water samples was based on molarity differences as well as on cationic content differences (Chapter 2, part 1, page - 52 -). They are also consistent with data acquired by Teillet *et al.* (2009b) since these authors also demonstrated that waters with very low TDS (equivalent to 2.05 mM) elicit a higher taste than water samples with a medium TDS (equivalent to 10.2 mM).
3.2.2. Chlorine flavour intensity

An ANOVA was performed on intensities reported on the chlorine flavour scale. This analysis evidenced a strong effect of chlorine level \(F(1, 1263)=322.6, p<0.0001\) but also interactions of this factor with both molarity \(F(1, 1263)=4.2, p=0.04\) and ionic pattern \(F(7, 1263)=2.3, p=0.02\) (see Table 8). As expected, non-chlorinated samples have a lower intensity rating \((M=2.5, SD=0.6)\) than chlorinated samples \((M=5.2, SD=0.6)\) \((p<0.001)\).

Results of this global modelling also evidence a significant effect of molarity \(F(1, 1263)=9.3, p=0.002\) and interactions between molarity and both ionic pattern \(F(7, 1263)=2.7, p=0.01\) and chlorine level \(F(1, 1263)=4.2, p=0.04\) as already mentioned.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pH)</td>
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<td>1263</td>
<td>3.4</td>
<td>0.07</td>
</tr>
<tr>
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<td>0.002</td>
</tr>
<tr>
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<td>1263</td>
<td>0.2</td>
<td>0.64</td>
</tr>
<tr>
<td>(Chlorine\ level)</td>
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<td>1263</td>
<td>322.6</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Molarity * (Chlorine\ level)</td>
<td>1</td>
<td>1263</td>
<td>4.2</td>
<td>0.04</td>
</tr>
<tr>
<td>Ionic pattern</td>
<td>7</td>
<td>1263</td>
<td>1.1</td>
<td>0.39</td>
</tr>
<tr>
<td>Ionic pattern * (Molarity)</td>
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<td>1263</td>
<td>2.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Ionic pattern * (Chlorine\ level)</td>
<td>7</td>
<td>1263</td>
<td>2.3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 8: Results of analysis of variance on chlorine flavour (retronasal) intensity performed with a mixed linear model: Test of fixed effects using type 1 error mean square
Considering the high effect of chlorine level on chlorine flavour perception, we conducted separate ANOVA for non-chlorinated and chlorinated samples.

The model estimated for non-chlorinated samples (Table 9) evidences that none of the factors tested likely explain chlorine flavour intensity. None of the non-chlorinated samples was discriminated on this scale (Figure 8). This means that panellists did not report the water taste on this scale and that they did not confuse the two attributes. However, one can notice that mean chlorine flavour intensity for non-chlorinated samples is relatively high (M=2.5).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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<td>623</td>
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<td>0.76</td>
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<td>Molarity</td>
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<td>623</td>
<td>0.38</td>
<td>0.54</td>
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<tr>
<td>Molarity * pH</td>
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<td>623</td>
<td>0.07</td>
<td>0.80</td>
</tr>
<tr>
<td>Ionic pattern</td>
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<td>623</td>
<td>0.48</td>
<td>0.85</td>
</tr>
<tr>
<td>Ionic pattern * Molarity</td>
<td>7</td>
<td>623</td>
<td>0.91</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Table 9: Results of analysis of variance on chlorine flavour (retronasal) intensity of chlorinated and non-chlorinated samples; ANOVA performed with a mixed linear model; Test of fixed effects using type 1 error mean square
The ANOVA model computed for chlorinated samples evidences a significant effect of molarity ($F_{(1, 619)}=3.8$, $p=0.05$) and interactions between molarity and both free chlorine ($F_{(1,619)}=5.2$, $p=0.02$) and ionic pattern ($F_{(7, 619)}=2.9$, $p=0.005$) (Table 9). Samples with a high molarity level tended to have a lower chlorine flavour intensity ($M=4.9$, $SD=0.5$) than samples with a low molarity level ($M=5.4$, $SD=0.5$) ($p<0.07$). Thus, separated analyses of variance were conducted for each molarity level. At low molarity level, the modelling results indicates that free chlorine ($F_{(1, 302)}=10.7$, $p=0.001$) and pH ($F_{(1, 302)}=5.57$, $p=0.02$ ; Table 10) are the only significant factors. Chlorine flavour intensity increases as free chlorine increases and pH decreases (Figure 9). The effect of pH is identical to the one observed in the orthonasal condition and relies on the pH-driven equilibrium between associated and dissociated form of hypochlorous acid. It is also logical that chlorine flavour intensity increases when free chlorine amount increases since free chlorine reflect the amount of non-combine hypochlorous acid. It also appears that pH and free chlorine amount tend to be correlated ($r=-0.67$, $p=0.07$), but this correlation is difficult to explain since the amount of free chlorine reflect both HOCl and ClO\(^{-}\) concentration. This result only underline that samples’ chlorination was not exactly identical for all the water samples produced.
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| pH | 1 | 302 | 5.8 | 0.02 |
| Free chlorine | 1 | 302 | 10.7 | 0.001 |
| Total chlorine | 1 | 302 | 0.0 | 0.88 |
| Ionic Pattern | 7 | 302 | 1.7 | 0.12 |

### chlorinated samples (0.3 mg/L Cl₂) / High molarity

| pH | 1 | 302 | 0.24 | 0.63 |
| Free chlorine | 1 | 302 | 0.02 | 0.88 |
| Total chlorine | 1 | 302 | 0.01 | 0.91 |
| Ionic Pattern | 7 | 302 | 3.23 | 0.003 |

Table 10: Results of analysis of variance on chlorine flavour (retronasal) intensity of chlorinated samples at low (2.05mM) or high (10.2mM) molarity level; ANOVA performed with a mixed linear model; Test of fixed effects using type 1 error mean square

![Figure 9: Mean chlorine flavour intensity rating for chlorinated samples adjusted at low molarity (2.05 mM)](image)

Black bars represent chlorine flavour intensity means estimated using the LSMEAN instruction of the MIXED procedure of SAS. Error bars reflect 95% confidence interval. Green line represents free chlorine whereas blue line represents pH of the samples.

At the high molarity level, the ANOVA model computation indicates that ionic pattern is the only significant factor \(F(1, 302)=3.23, p=0.003\; \text{Table 10}\). Figure 10 shows chlorine flavour intensity means obtained for the different ionic patterns. The water with a high proportion of \(\text{Ca}^{2+}\) elicits significantly lower chlorine flavour intensity than the water with a high proportion of \(\text{Na}^+\) and \(\text{Cl}^-\) and the water with a high proportion of \(\text{HCO}_3^-\). To summarize, samples with
high proportion of Ca\textsuperscript{2+} seem to have lower chlorine intensity rating than samples with high proportion of Na\textsuperscript{+}.

![Figure 10: Mean chlorine flavour intensity rating for chlorinated samples adjusted at high molarity (10.2 mM)](image)

Black bars represent chlorine flavour intensity means and were estimated using the LSMEAN instruction of the MIXED procedure of SAS. Error bars reflect 95% confidence interval.

The above results demonstrated that water taste is driven by sodium content and molarity in a range of variation reconcilable with tap water. Low molarity and high Na\textsuperscript{+} content elicit higher taste.

Concerning chlorine perception, we evidenced through chlorine odour (orthonasal) measurement that physicochemical parameters of water matrix are likely to modify chlorine perception. Thus, pH has been identified as a significant factor at low molarity level. At high molarity level, high proportions of Na\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-} seem to be associated to lower chlorine perception.

Once in mouth, namely during retronasal chlorine flavour measurements, other factors may drive chlorine flavour perception. Thus, it has been demonstrated that waters adjusted at a molarity of 2.05 mM obtained higher chlorine flavour intensity rating than waters adjusted at a higher molarity (10.2 mM). In a previous study, using bottled waters, we found in a higher range of molarity levels (>25-35 mM) that water samples with the higher molarity were judge as more chlorinated (Chapter 2, publication 6, page - 102 -), but in the range of molarity 2.05-10.2 mM, we failed to find significant difference in chlorine flavour perception. Taken together, these findings suggest that waters with a medium molarity level (10.2 mM) and chlorinated at a level compatible with tap water (0.3 mg/L) may develop a lower chlorine flavour intensity as compared to waters with lower (2.05 mM) of higher molarity levels (> 25-35 mM). Such an
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Observation indicates that water taste perception may affect chlorine flavour perception. Indeed, our study (preliminary study) as well as studies of others (Teillet et al. 2009b) evidenced that water taste is lower at a medium level of molarity as compared to low or high molarity levels. Nevertheless, deeper analysis conducted on water taste intensity suggests that water with a high content of Na\(^+\) has a higher water taste whatever the molarity level. Besides, samples with high proportion of Ca\(^{2+}\) seem to develop lower chlorine intensity than samples with high proportion of Na\(^+\). These observations strengthen the idea that water taste could be a driven factor of chlorine flavour perception. To explore this hypothesis, we conducted another ANOVA on chlorine flavour intensity of the chlorinated samples. As for the other analyses a mixed linear modelling was applied with judge and repetition as random factors. Sensory data, namely chlorine odour, water taste and their interaction were introduced in the model as covariates. The results showed that chlorine odour intensity is surprisingly not linked to chlorine flavour intensity of chlorinated samples (\(F_{(1, 637)}=0.25, p=0.61\)). In the same way, neither water taste nor the interaction between water taste and chlorine odour reach significant level (respectively \(F_{(1, 637)}=2.38, p=0.12\) and \(F_{(1, 637)}=2.08, p=0.15\)). Thus, none of these factors are likely to account for chlorine flavour perception, suggesting that in-mouth interactions could occur (e.g. temperature, reaction of free chlorine, saliva mineral content or pH).

4. Conclusion

This study points out the impact of ionic pattern and molarity on chlorine perception evaluated through the nose or in the mouth. Moreover, the results evidenced that water taste is driven both by molarity and sodium content. As far as chlorine odour perception is concerned, we evidenced an influence of water minerals content suggesting physicochemical mechanisms on hypochlorous acid release. Chlorine flavour intensity when perceived via retronasal route is also driven by molarity. However, chlorine flavour intensity could be linked neither to chlorine odour intensity nor to water taste, suggesting that in-mouth biophysicochemical interactions could occur. Further investigations are needed to measure content of HOCl in the headspace of various chlorinated water samples but also to measure HOCl concentration in the mouth. Such analysis would allow to better understand mechanisms occurring during chlorinated tap water consumption which are especially difficult to assess due to the low intensity levels. Even if the mechanisms are not fully understood, our data revealed that chlorine flavour intensity increases as molarity decreases. This result has practical consequence for tap water suppliers. Treatments such as carbonate removal lead to a reduction of the total amount of mineral and thus would have for consequence an increase of chlorine flavour intensity.
4. Partial discussion:

This chapter aimed to investigate the ability of water mineral matrix constituents to mitigate chlorine flavour perception. Before starting to investigate cross-modal interactions, we investigated first the determinants of water taste. If the impact of mineral amount was clearly evidenced in several studies (e.g. Bruvold and Gaffey 1969; Bruvold 1970; Teillet et al. 2009a), individual impact of ions in water was not taken into account. Indeed, author working on water most often use TDS to traduce impact of mineral content. Even if very commonly used to express water mineral content, TDS is not the most accurate variable to study the taste of water. Indeed, TDS correspond to the quantity, expressed in mass, of all minerals dissolved in water whatever their quality and their molecular weight. In the framework of water taste, TDS rely on osmolarity detection but also on other sensory mechanism such as adaptation to saliva. In our studies, we use molarity instead of TDS in order to account for the real quantity of molecules that could actually reach the receptors. We found that molarity is the most important factor affecting water taste. This result was also evidence using TDS. However, the use of molarity enabled us to consider different mechanisms potentially implied in mineral-induced taste perception. Thus, we determined that beyond molarity, the nature of ions influenced the water taste: discrimination between water varying in mineral content is mainly based on the composition in cations. In our studies, we especially focused on tap water composition. Therefore, following the determination of main typologies of tap and bottled waters, we evidenced that the taste of tap water is mainly driven by its molarity and its Na\textsuperscript{+} content.

Beyond water taste, this chapter was dedicated to explore putative aroma-taste perceptual interactions as a lever to reduce chlorine flavour perception. Our results evidenced that chlorine flavour perception varied according to water mineral matrix composition. However, these interactions seem to be due to physicochemical effects rather than to sensory interactions. Our data highlighted the influence of water pH, which obviously depend on water mineral content, and could modulate the hypochlorous acid stimulus concentration in the headspace and consequently affect the chlorine odour/flavour perception. Our findings also suggested that in-mouth mechanisms may occur (e.g. influence of saliva composition). However, the nature of these mechanisms cannot be inferred from our data. Further measurements of HOCl content and chlorinated species in headspace and in vivo mouth-
Chapter II Part 2 – Cross modal interactions affection chlorine flavour perception – Partial discussion

space are especially needed. Such kind of measurements would offer a more comprehensive understanding of the implied perception mechanisms.

An important outcome of this study is the link between chlorine flavour perception and molarity. Our results indicated that the perception of chlorine flavour is the lowest for waters containing a medium amount of minerals (10.2 mM). If this level is medium for bottled water, most of the drinking waters were found below this limit. For lower molarities (likely for tap water) or higher molarities (unlikely for tap water) the perception of chlorine was found to increase. This finding has practical consequences for tap water supplier. Indeed, another frequent complaint of consumers, as far as tap water in concerned, is the hardness of water. This water hardness is directly linked to a high tap water overall molarity. To remedy this problem, suppliers often various softening processes (i.e. lime softening, Sodium hydroxide softening and Electrochemical water softening). These treatments have for consequence a reduction of water molarity as well as a change in ionic pattern of water. Such, a reduction of water total ionic content would have for consequence an increase of chlorine flavour perception and as well as a more pronounced water taste. In such a context, it is important to assess consumer preference and acceptability for such composition modifications. Indeed, since consumers prefer neutral waters to drink and depreciate chlorine flavour, acceptability is supposed to decrease for softened waters. All these consequences of softening need to be considered to ensure consumers’ satisfaction. Research has to be done to understand basis of consumer decision making and consequences on supply image and consumer willingness to pay for this multipurpose product.
Chapter 3.

Using perceptual odour interactions to reduce chlorine odour perception
Chapter III – Using perceptual odour interactions to reduce chlorine odour perception –
Introduction

1. Introduction

One of the objectives of the PhD is to explore chlorine flavour perceptual neutralisation levers. The previous chapter aimed to determine to what extent mineral matrix of water can mitigate chlorine flavour perception. In the current chapter, we investigated the possibility to reduce chlorine odour using other odorants at peri-threshold concentration.

Odours we smell in every day life are most often due to complex mixtures of odorants. The odour of such complex mixtures does not rely on the simple addition of each odorant perception. Olfactory information due to these odorants is processed at different levels of the olfactory system and is subjected to several interaction mechanisms resulting in odour synergy, masking or fusion (Berglund et al. 1976; Frijters 1987). Most of the studies conducted on odorant mixtures rely on binary mixtures. Berglund et al. (1976) propose classifications of qualitative and quantitative effects occurring in binary mixture. They classify mixtures according to qualitative perception. Indeed, they distinguish mixtures eliciting homogenous and heterogeneous perception. Heterogeneous perception characterises binary mixtures in which the two components are still perceived. On the opposite, homogeneous perception characterizes mixtures in which the odours of components blend and give way to the emergence of a new odour.

Cain and Drexler (1974) describe the relation between the intensity of a mixture and the intensities of the unmixed components (Table 1). Thus, complete addition describes a mixture in which intensity is equal to the sum of intensities of the unmixed components. Hyper-addition refers to a mixture in which intensity is higher than the sum of intensities of the unmixed components. And finally, hypo-addition refers to a mixture in which intensity is lower than the sum of intensities of the unmixed components. Several cases exist for hypo-addition. When intensity of the mixture is lower than the lowest intensity of unmixed components, the term compensation (subtraction) is used. When intensity of the mixture is comprised between the minimum and maximum intensities of unmixed components, the term used is compromise. Finally, the term partial addition is used to describe a mixture in which intensity is higher than the intensity of the most intense component but lower than the sum of intensities of the unmixed components.

Berglund et al. (1976 ) propose to apply these definitions to homogeneous mixtures and propose other terms for heterogeneous mixtures. Synergy is used when the intensity of a compound is higher in the mixture than perceived alone. On the opposite, antagonism (or masking) is used when the intensity of a compound is lower in the mixture than perceived
Chapter III – Using perceptual odour interactions to reduce chlorine odour perception –

Introduction

alone. And finally, independence is used when intensity of component is equal when perceived in or out of the mixture (Table 1).

<table>
<thead>
<tr>
<th>Unmixed components</th>
<th>Component A</th>
<th>Component B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneous mixture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete addition</td>
<td>$I_M = I_A + I_B$</td>
<td></td>
</tr>
<tr>
<td>Hyper addition</td>
<td>$I_M &gt; I_A + I_B$</td>
<td></td>
</tr>
<tr>
<td>Partial addition</td>
<td>$I_M = I_A$</td>
<td></td>
</tr>
<tr>
<td>Hypo addition</td>
<td>$B &lt; I_M &lt; I_A$</td>
<td></td>
</tr>
<tr>
<td>Compromise</td>
<td>$I_M &lt; I_B$</td>
<td></td>
</tr>
</tbody>
</table>

| Heterogeneous mixture | Synergism for A / Independence for B | $I_{MA} > I_A$ | $I_B$ |
|                       | Independence for A / Independence for B | $I_{MA} = I_A$ | $I_B$ |
|                       | Antagonism for A / Independence for B  | $I_{MA} < I_A$ | $I_B$ |

Table 2: Effects on perceived intensity for binary mixtures (Thomas-Danguin 1997)

$I_{A(B)}$: intensity of component A(B). $I_M$: intensity of the homogeneous mixture. $I_{MA(B)}$: Intensity of component A(B) in the heterogeneous mixture.

The purpose of this part of the PhD thesis is to identify molecules which could neutralise chlorine flavour perception either by modification of its perceived quality or by reduction of its intensity. Therefore, the mixture of chlorine odour with the neutralising agent should give rise to compromise, subtraction or antagonism (masking) effects. As soon as the neutralising molecule may be added to drinking water, this compound should not bring a marked flavour to water. Thus, this compound has to be efficient at peri-threshold concentrations.

A few studies on odorant mixtures investigated the impact of odorant at sub-threshold concentrations. Guadagni et al. (1963) evidenced additive mixture as a mixture in which it is possible to perceive an odour even if individual components are all present at sub-threshold concentrations. Atanasova et al. (2005a), Atanasova et al. (2005b) and Ishii et al. (2008), working on compounds present in wine, evidenced the impact of sub- and peri-threshold woody components on the perception of supra threshold fruity odorants. These authors observed synergism of the fruity odour when mixed with sub-threshold woody component. On the opposite, they observed masking in mixtures of fruity odour with supra-threshold woody component.
Chapter III – Using perceptual odour interactions to reduce chlorine odour perception –

Introduction

If most of the few studies which deal with sub-threshold concentrations of one or more components in mixture observed additive effects, the most common effect reported in odorant mixtures is hypo-addition (Berglund and Olsson 1993; Olsson 1994). All types of hypo-addition have been encountered. Cain and Drexler (1974) identified compromise: the less intense compound decreases the intensity of the most intense odorant in the mixture. Moskowitz and Barbe (1977) also describe mixture in which odour is less intense than that of the less intense unmixed component. Thus, our aim was to identify such kind of interactions occurring between chlorine and neutralising odorants at peri-threshold level.

As previously mentioned, the neutralising compound is intended to be used on network. For that reason, this compound has to reduce chlorine flavour perception without conferring a marked flavour to water and has also to meet several criteria associated with its potential use. First of all, it has to be food grade. It has to be water-soluble between 4°C and 24°C. This range corresponds to the temperatures of water encountered on distribution networks. Above all, no physico-chemical reaction must occur between compounds likely to reduce chlorine odour and both chlorine and materials presents on water network (pipes, etc.). Once solubilised in water, this neutralizing compound has not to modify the HOCl content which is the efficient form of chlorine in water as far as disinfection is concerned (Connell 1996).

To select compounds likely to meet this list of criteria, a screening was conducted in partnership with Robertet SA, a flavour and fragrance company. This company has screened more than 170 compounds. The first step of the screening consisted to verify that no physico-chemical reaction occurred between compounds of interest and chlorine in water. This was done through comparison of free and total chlorine content for a reference solution chlorinated at 0.3 mg/L Cl₂ and the solution in which the compound of interest was added at peri-threshold concentrations. To mimic the possible delay between water treatment and water delivery at tap, measurements were performed at the time of preparation but also 24H after preparation. Based on results obtained from these tests, 109 compounds were discarded. Then, the 61 remaining compounds were submitted to a first sensory test performed by 6 aromaticians of the company. This test consisted in 2 paired comparison tests aiming to compare chlorine flavour intensity for a solution of chlorine adjusted at 0.3 mg/L Cl₂ and the same solution containing the compound of interest at peri-threshold concentration; this concentration being determined by preliminary assays. Based on these tests, 29 compounds were selected to participate in a second phase of sensory testing. Once again, 6 aromaticians performed two triangular tests aiming to determine whether the solution containing the aromatic compound was perceived as less chlorinated or not. In fine, 16 aromatic solutions were found less chlorinated than the reference in 8 out of 12 trials.
Chapter III – Using perceptual odour interactions to reduce chlorine odour perception –

Introduction

(p<0.05). The odorant included in these solutions were selected to be evaluated in a final screening test.

This final test aimed to validate the chlorine flavour masking ability of the selected compounds. It was conducted in the framework of the PhD work and is presented in the first study reported in this chapter (publication 8, page -144-). It consisted to assess the potency of the selected compounds to reduce chlorine flavour perception and/or to increase liking or acceptability of chlorinated water. These evaluations have been conducted with tap water consumers and bottled water consumers to assess the impact of such addition on consumers with different drinking water habits and representations. Systematic measurements of chlorine concentration have been conducted on the solutions delivered during the study in order to verify the absence of physico-chemical reaction between chlorine and the compounds added. The second study presented in this chapter (publication 9, page 155) aimed to investigate, using a methodology that ensure an absence of chemical reaction between odour component, binary mixtures of chlorine with a selection of compounds to determine optimal proportions of the neutralising agents to reduce chlorine odour. This chapter ends with a short discussion of the results.
2. Publication 8: Screening of odorants able to reduce chlorine flavour perception

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1. Introduction

Sixteen odorants were preselected by the Robertet SA company following a screening procedure which aimed to evaluate food grade odorants that have the ability to reduce chlorine flavour perception. The purpose of the current study is to validate the efficiency of these compounds to actually reduce chlorine flavour perception in water to be tasted. Additionally, liking and acceptability scores were recorded. To do so, a consumer study was conducted with a group of exclusive tap water consumers and a group of exclusive bottled water consumers. Indeed, in a previous study (Puget, Beno et al. 2010) we observed a difference between tap water consumers and non-consumers for liking and acceptability of chlorinated solutions. Thus, addition of a compound to chlorinated water could be differentially accepted by tap water consumers and bottled water consumers.

2. Material and methods

2.1. Subjects

63 consumers were recruited on the basis of their water consumption. Two groups of consumers were constituted. Thirty six consumers (14 women, 22 men; 40 ± 15 years old) were included in the tap water consumer group (people who daily drink chlorinated tap water without using any device or process to reduce chlorine flavour). Twenty seven consumers (16 women, 11 men; 41 ± 14 years old) were included in the tap water non-consumer group (people who do not drink tap water and declared themselves to be exclusive bottled water consumers).

The participants had never participated to sensory studies. They signed an informed consent form but the aim of the experiment was not revealed. They were asked not to modify their water consumption during the study and to avoid smoking, drinking and eating at least one hour before each session and to avoid using perfume the day of the test. Subjects were paid for their participation (10 € per hour).
2.2. Water samples

Chlorinated water samples were obtained by adding sodium hypochlorite (NaOCl 15%, RECTAPUR, VWR international, France) to Evian water (La Bourgogne, Dijon, France). Evian water was purchased in 1 L glass bottles from the same lot. The concentration of chlorine was adjusted at 0.3 mg/L Cl\(_2\) for all samples.

A total of 17 water samples were prepared. Sixteen samples contained chlorine and a putative neutralising compound provided by Robertet SA (Table 1). The last sample contained chlorine only and was used as reference. Because of chlorine’s high volatility and degradation by sunlight (UV), solutions were daily prepared and stored according to the procedure described in Puget, Beno, et al. (2010). The expected chlorine content of the delivered samples was controlled by free chorine measurement performed through a procedure (pocket colorimeter II, Hach Lange) adapted from the DPD protocol for spectrophotometry (APHA-AWWA-WEF 1998). This parameter was controlled daily in each flask just after solution preparation and at the end of each sensory session.

<table>
<thead>
<tr>
<th>Water sample</th>
<th>Compound</th>
<th>Concentration used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water 1</td>
<td>Å</td>
<td></td>
</tr>
<tr>
<td>Water 2</td>
<td>2-methylbutyric acid</td>
<td>1 ppm</td>
</tr>
<tr>
<td>Water 3</td>
<td>2-methylpyrazine</td>
<td>1 ppm</td>
</tr>
<tr>
<td>Water 4</td>
<td>Acetic acid</td>
<td>1 ppm</td>
</tr>
<tr>
<td>Water 5</td>
<td>Acetoin</td>
<td>400 ppb</td>
</tr>
<tr>
<td>Water 6</td>
<td>Cinnamaldehyde</td>
<td>32 ppb</td>
</tr>
<tr>
<td>Water 7</td>
<td>Cinnamic acid</td>
<td>14 ppb</td>
</tr>
<tr>
<td>Water 8</td>
<td>Dimethyl benzyl carbinol</td>
<td>1 ppm</td>
</tr>
<tr>
<td>Water 9</td>
<td>Ethyl acetate</td>
<td>1 ppm</td>
</tr>
<tr>
<td>Water 10</td>
<td>Ethyl butyrate</td>
<td>3.3 ppb</td>
</tr>
<tr>
<td>Water 11</td>
<td>Isoamyle acetate</td>
<td>2 ppb</td>
</tr>
<tr>
<td>Water 12</td>
<td>Methyl-2-methyl butyrate</td>
<td>7.5 ppb</td>
</tr>
<tr>
<td>Water 13</td>
<td>n-Butanol</td>
<td>1 ppm</td>
</tr>
<tr>
<td>Water 14</td>
<td>Phenylethyl alcohol</td>
<td>121 ppb</td>
</tr>
<tr>
<td>Water 15</td>
<td>Propyl butyrate</td>
<td>38 ppb</td>
</tr>
<tr>
<td>Water 16</td>
<td>Styrryl acetate</td>
<td>200 ppb</td>
</tr>
<tr>
<td>Water 17</td>
<td>Whiskey lactone</td>
<td>1.7 ppb</td>
</tr>
</tbody>
</table>

Table 1: Composition of the 17 samples evaluated in sensory sessions.

Each sample was chlorinated at 0.3 mg/L. 16 samples also contained an aromatic compound (putative neutralising compound).

2.3. Sensory procedure

Consumers were invited to participate in two sensory sessions which were conducted in a room dedicated to sensory analysis following HACCP and Research Quality Insurance Standards. Consumers were placed in separate booths and their responses were collected using a software dedicated to sensory analysis (FIZZ, Biosystèmes, Couternon, France).

The first session lasted 1 hour and was dedicated to assess the potency of selected aroma compounds to reduce chlorine flavour perception. This was done through paired comparison
tests (AFNOR 2007c). This comparison consisted in the simultaneous presentation of two samples: one being the reference (Evian water chlorinated at 0.3 mg/L Cl₂) whereas the other one was a water solution chlorinated at the same level but containing one of the 16 neutralising compounds at the concentration indicated in table 1. Consumers had to compare chlorine flavour intensity of the water samples and were asked to indicate which one had the highest chlorine flavour intensity. A last paired comparison was performed to compare the reference solution chlorinated at 0.3 mg/L Cl₂ with non-chlorinated Evian water. This last paired comparison was used as a reference of chlorine flavour neutralising effect.

The second session lasted 1.5 hour and was dedicated to the evaluation of liking and acceptability for the 16 chlorinated solutions containing one of the neutralising compounds and for the reference (Evian chlorinated at 0.3 mg/L Cl₂). This session was divided into two blocks; the first one was dedicated to liking rating and the second to acceptability judgements. Within the first block, liking score for each sample was rated on a linear scale from “I don’t like this sample” to “I like this sample”. Within the second block, consumers were asked to answer yes or no to the question: “If this water was daily delivered to your tap, would you drink it?”.

For each evaluation, consumers received 10 ml of water sample presented in plastic cups coded with a three-digit number. For each evaluation, sample order was different and followed a Williams Latin square. Subjects had to wait at least 120 seconds between two successive evaluations. They were instructed to rinse their mouth with Evian water during this inter-trial interval.

2.4. Data analysis

2.4.1. Physicochemical data

Physicochemical controls were performed to determine whether compound addition modifies actual chlorine content of the samples. Free chlorine measurements were submitted to an ANOVA with compound as factor (GLM procedure of SAS). Post hoc comparisons of means were performed to compare the mean of the control with means obtained for solutions containing neutralising compounds. This was done using Dunnett-Hsu adjustment for multiple comparisons.

2.4.2. Paired comparisons data

For each paired comparison test, two types of answers were possible. The first one was the situation where the chlorine intensity of the sample (Evian water + chlorine + neutralising compound) was higher than the reference (Evian water + chlorine): I\textsubscript{chlorine+neutralisant} > I\textsubscript{chlorine}. 

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This answer was coded with a 0. The other possible response, coded with a 1, appeared when the chlorine intensity of the reference was higher than the intensity of the sample: 
\[ I_{\text{chlorine}} > I_{\text{chlorine+neutralisant}}. \]

A first analysis was conducted to determine whether the proportion of panelists answers for which \( I_{\text{chlorine}} > I_{\text{chlorine+neutralisant}} \) could be explained by the consumer group, the nature of the neutralising compound and the interaction between these factors. Individual responses obtained for each comparison were analysed through the Generalized Equation Estimation for binary data (GENMOD procedure of SAS) with subjects as a repeated effect (Zeger et al. 1988).

Then, for each comparison, the number of responses where the chlorine intensity was higher for the sample as compared to the reference was compared to the number of responses obtained by chance with a probability of 5% (\( p=0.05 \)). This probability is distributed as a binomial law \([B(n=63, p_0=\frac{1}{2})]\) and the corresponding number of answers was calculated using a SAS® macro, BINRISK developed by Schlisch (1993). A Bonferroni correction for multiple comparisons was applied.

### 2.4.3. Liking scores

Liking rating data were submitted to an ANOVA (Analysis of Variance) following a mixed linear modelling with subjects as random factor (MIXED procedure of SAS). The consumer group, the type of compound and their interaction were used as factors. Post-hoc comparison of least square means was performed with Dunnett-Hsu adjustment.

### 2.4.4. Acceptability

Acceptability data consisted in yes or no answers and were analyzed through the Generalized Equation Estimation for binary data (GENMOD procedure of SAS) with consumers as a repeated effect (Zeger et al., 1988). The consumer group, the type of compound and their interaction were used as factors. Acceptability differences were determined through \( \chi^2 \) calculations (FREQ procedure of SAS).

### 3. Results and discussion

#### 3.1.1. Physicochemical controls

Free chlorine measurements were performed to validate the absence of physicochemical reaction between hypochlorous acid and the neutralising compound added in the solution to reduce chlorine perception. Results presented in Figure 1 evidenced that the chlorinated solution containing cinnamic acid had a significantly lower free chlorine level. This result
indicated that cinnamic acid chemically reacts with the hypochlorous acid chlorine agent which led to the reduction in free chlorine concentration in the water solution.

![Figure 5: Mean free chlorine concentration in chlorinated solutions including neutralising compound.](image)

Black bars represent free chlorine mean concentration for the water samples in which a neutralising compound was added. The red bar represents free chlorine mean for the reference solution which contained chlorine only. Error bars are 95% confidence interval. Significance level of the difference between the reference solution and the solutions containing an aromatic compound were estimated by mean comparison with Dunnett-Hsu adjustment (\(^*\) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).

### 3.1.2. Chlorine flavour intensity for chlorinated solutions including a neutralising compound

Sixteen paired comparison tests were performed to assess the efficiency of neutralising compounds to reduce chlorine flavour perception. Each test aimed to compare chlorine flavour intensity of a sample (chlorine + neutralising compound) to the reference solution chlorinated at 0.3 mg/L Cl\(_2\). A supplementary paired comparison test was performed to compare the reference solution with non-chlorinated Evian water. This comparison provided a reference level for chlorine flavour reduction.

Results are presented in Figure 2. A general estimation equation (GEE) was conducted to explain individual answers for which \(I_{\text{chlorine}} > I_{\text{chlorine+neutralisant}}\). The consumer group, the nature of neutralising compound and their interaction were used as factors and subjects as a repeated effect. Results evidenced a significant effect of the nature of the neutralising compound (\(Z_{(16,1071)} = 58.3; p < 0.0001\)). Neither the consumer group factor (tap water consumers vs. bottled water consumers) (\(t_{(1,1071)} = 0.38; p=0.54\)) nor the interaction between...
these two factors ($z_{(16,1071)} = 23.2; p=0.11$) were found to be significant. The lack of significance of the consumer group factor is not surprising. Indeed, we demonstrated in a previous study that tap water consumers and bottled water consumers did not differ in their chlorine sensitive neither at threshold nor at supra-threshold levels (Puget et al. 2010). The number of consumers estimating the reference solution to have higher chlorine flavour intensity than the sample including a neutralising compound is presented in Figure 2a whereas Figure 2b presents the inverse situation (sample more intense than reference). Results presented in Figure 2a indicated that none of the tested neutralising compound significantly reduced chlorine flavour perception. Nevertheless, it appears clearly that non-chlorinated Evian water was perceived as having a less intense chlorine flavour. Conversely, results presented in Figure 2b revealed that two compounds (2-methylpyrazine and whiskey lactone) enhanced chlorine flavour intensity.
Figure 2 answers obtained for the paired comparison tests.

Figure 2a presents the number of consumers for which the reference solution chlorinated at 0.3 mg/L Cl₂ was perceived as more chlorinated than the chlorinated sample solution containing the neutralising compound. Figure 2b presents the reverse situation (sample more intense than reference). On both figures, black bars represent the number of responses in each situation and for each neutralising compound. The red bar results of the comparison between the reference solution and non-chlorinated Evian water. Black lines represent the minimal number of answers to consider the difference between samples as significant (p=0.05). Significance of comparisons are reported on top of the histogram bars ((*) 0.05<p<0.1; * p<0.5; ** p<0.01; *** p<0.001).
3.1.3. Liking scores for chlorinated solutions including neutralising compounds

Consumers indicated their liking for the reference solution chlorinated at 0.3 mg/L Cl₂ and the chlorinated sample solutions including the neutralising compounds. To assess the effects of consumer group, nature of neutralising compound and their interaction as factors on Liking scores, data were computed according to an ANOVA procedure. Results revealed that the consumer group factor tended to be significant ($F_{(1, 61)}=3.93$, $p=0.06$). Indeed, tap water consumers had a higher liking ($M=5.4$, $SD=0.2$) than bottled water consumers ($M=4.7$, $SD=0.2$). This observation is in line with data previously acquired in the framework of the PhD showing that the two consumer groups differ in their liking and acceptability of chlorinated solutions (Puget et al. 2010). ANOVA results indicated that the nature of neutralising compound significantly influenced liking ($F_{(16, 976)}=2.93$, $p<0.001$) but not its interaction with the consumer group factor ($F_{(16, 976)}=0.9$, $p=0.6$). These results indicated that solutions have a different liking score depending on the neutralising compound (Figure 3) and that both groups of consumers gave similar responses. The sample containing Cinnamic acid was the only one to obtain a higher liking score than the reference solution. However, this sample was also the one with the lower residual, indicating possible reaction between chlorine and this compound. So, it seemed obvious that this lower chlorine residual was mainly responsible of the good neutralizing performance of this compound. However, mean comparison performed with Dunnett-Hsu adjustment indicates that this comparison was not significant ($t_{(976)}=1.9$, $p=0.4$).

The only neutralising compound able to induce a liking score significantly different from the reference is styryl acetate. Solution containing this compounds was indeed significantly less appreciated than the reference solution ($t_{(976)}=-3.4$, $p=0.02$).
Figure 3: Liking scores for chlorinated solutions including neutralising compounds.

Black bars represent liking mean for the water samples in which neutralising compounds were added. The red bar represents liking mean for the reference solution which does not contain chlorine only. The dotted line also indicate liking mean for this sample. Error bars are 95% confidence interval. Significance level of the difference between the reference solution and the solutions containing a neutralising compound were estimated by mean comparison with Dunnett-Hsu adjustment (\((^*) \) 0.05<p<0.1; \(* \) p<0.5; \(** \) p<0.01; \(*** \) p<0.001).

3.1.4. Acceptability scores for chlorinated solutions including neutralising compounds.

Acceptability score for the reference solution chlorinated at 0.3 mg/L Cl\(_2\) and the chlorinated sample solutions including one of the neutralising compounds were assessed using the following question. “If this water was daily delivered to your tap, would you drink it?” Yes or No answers were analyzed through the Generalized Equation Estimation with consumers as a repeated effect. The consumer group, the nature of compound and their interaction were used as factors. Acceptability difference between compared samples was determined through \(\chi^2\) calculation (FREQ procedure of SAS).

Results revealed that the nature of the neutralising compound significantly influenced acceptability mean score (\(z_{(16,1071)} = 28.6; p = 0.03\)). Similarly, the group of consumer factor was found to be significant (\(z_{(1,1071)} = 3.7; p = 0.05\)). As previously demonstrated (Puget et al. 2010), tap water consumers are more inclined to accept chlorinated solution as drinking
water. Thus, 70% of the tap water consumers would accept to daily drink the chlorinated solutions against only 48% of the bottled water consumers. In the same way we compared here the proportion of consumers likely to accept the chlorinated solution whatever the neutralising compound added. We found that the proportion of bottled water consumers remained unchanged (50%) whereas the proportion of tap water consumers decreased from 70% to 61%. This suggests that the modification of water flavour by addition of neutralising compounds would have as effect to decrease acceptability for tap water consumers. The interaction between the factor consumer group and the factor nature of neutralising compound is not significant ($Z_{16,1071} = 15.1; \ p = 0.5$). Therefore the acceptability of chlorinated solutions was evaluated for the whole set of consumers. Results presented in Figure 4 evidence that, in mean, adding a neutralising compound to chlorinated water tended to decrease acceptability. Only one compound however altered significantly the acceptability: methyl-2-methyl butyrate ($\chi^2_{1, 126}=16.8, \ p<0.001$) significantly decreased sample acceptability as compared to the reference (chlorinated Evian water).

![Figure 4: Acceptability scores of chlorinated solutions including neutralising compounds.](image-url)

Black bars represent the number of consumers who would drink at tap one of the water samples including one of the neutralising compounds. The red bar represents the number of consumers who would accept to drink the reference solution which include chlorine only. Error bars are 95% confidence interval. Significance level of the difference between the reference solution and the solutions containing a neutralising compound were estimated by Chi2 tests ($* 0.05<p<0.1; \ * p<0.5; \ ** p<0.01; \ *** p<0.001$).
4. Conclusion

The first outcome of this study is the confirmation that tap water consumers and bottled water consumers do not differ in their sensitivity to chlorine but differ in their liking for chlorinated water and their acceptability to drink chlorinated water (Puget et al. 2010). Concerning the ability of aroma compounds to neutralise chlorine flavour at a perceptual level, the results obtained in this study do not allow to find any compound able to reduce chlorine flavour perception or to increase chlorinated water liking or acceptability. Conversely, this study highlighted 2 aroma compounds that enhance chlorine flavour perception (i.e. 2-methylpyrazine and whiskey lactone). This result confirms the existence of perceptual interactions as already demonstrated for example in the case of the enhancement of fruity notes by whiskey lactone at sub-threshold concentrations and suppression at higher concentrations (Atanasova et al. 2005c; Atanasova 2004; Ishii et al. 2008). Most of cases published in the literature and dealing with perceptual interactions in odour mixtures reported that sub-threshold concentrations of a compound in the mixture induced an enhancement (e.g. Guadagni et al. 1963). Nevertheless, some studies, usually reported in patents, reported a masking of some compounds added at sub- or peri-threshold concentration levels (Laffort 1995; Schleppnik and Vanata 1977). The main issue in these documents is that the mechanism at the origin of masking is completely unknown. As a consequence it is quite difficult to perform an efficient screening of molecules that may have masking abilities. At the moment, the screening strategy relies mainly on “finding a needle in a haystack”. If very recent studies have highlighted antagonism both at olfactory receptor and perceptual levels (Spehr et al. 2004; Brodin et al. 2009), current knowledge on the olfactory coding does not allow to predict masking activity on the basis of chemical structures or odorants’ perceptual properties.
3. Publication 9: Extensive study of chlorine odour neutralisation

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3 CIRSEE, Suez Environnement, Le Pecq, France.
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1. Introduction

A first screening has been conducted to identify compounds likely to reduce chlorine flavour perception. From 170 odorants initially tested, the 16 most promising compounds were further submitted to a consumer study. However, this consumer test showed that none of the tested compound was found to have the ability to reduce chlorine flavour perception without altering free chlorine concentration into the water solution.

Compounds chosen for the initial screening were selected in order to cover a large variety of functional groups. Another option would be to choose compounds already known to be implied in perceptual interactions within odour mixtures. Olfactory interactions are likely to occur at different levels of the olfactory process. Berglund et al. (1976) and Frijters (1987) describe 3 main levels of interaction. The first level is called pre-sensory and relies on the physicochemical interactions between compounds. This level must not be considered here since it is not reconcilable with the use of the selected compounds in drinking water network. Indeed, as already mentioned, any chemical reaction with active chlorine is not suitable. The second level concerns the earlier stages of the olfactory processing. For example, antagonism between compounds at the receptor and the neuroreceptor levels was reported (Sanz et al. 2005; Duchamp-Viret et al. 2003). Interactions at the receptor level were also found to be reflected at the perceptual level (Spehr et al. 2004; Brodin et al. 2009). Finally, the third level of interaction is cognitive and relies on the integration of olfactory information with other information from different part of the central nervous system and especially from gustatory or somatosensory systems. Thus, cognitive processes involved in flavour are also involved in odour perception. For example, Stevenson (2001) demonstrated that odour pairs experienced together in a mixture should also be less discriminable than odour presented and encoded separately. Specific associations encoded during experiences with odour are thus likely to be the basis of perceptual interactions between odour components.
Chapter III – Using perceptual odour interactions to reduce chlorine odour perception – Publication 9

The objective of this last study was to perform an extensive study on chlorine odour masking using 3 well selected compounds and to investigate several odour intensity ratios of both chlorine and putative neutralising compound.

The first neutralising compound selected was cinnamaldehyde. It has been chosen on the basis of possible cognitive interactions with chlorine odour. Indeed, in carbonated beverages CO$_2$, which activates the trigeminal system, is associated to several aroma compounds such as cinnamon. Besides, we reported that hypochlorous acid (HOCl) could activate the trigeminal system (Chapter 1, publication 2, page - 30 -). Thus, we hypothesised that an association between the cinnamon odour of cinnamaldehyde and a weak nasal irritation engaged by HOCl through the trigeminal system activation could create a cross modal interaction that would result in a decrease of the chlorine odour also induced by HOCl. Following a similar hypothesis, salt-associated odours could evoke the salty taste (Lawrence et al. 2009). We found (Chapter 2, publication 7, page - 116 -) that sodium ions, which are responsible for the salty taste, confer a taste to water. Even if the impact of sodium ions on chlorine flavour of chlorinated water was not found to be very clear, we hypothesised here that the salty sensory dimension could mask the chlorine odour dimension. Thus, 3-butyldeneptaldehyde has been selected as a putative neutralising agent because it evokes a celery odour, namely a salt-associated odour (Lawrence et al., 2009).

Finally whiskey lactone has been selected because it was identified in the screening test as a compound which odour could perceptually interact with, even if enhance, chlorine flavour perception. Previous studies demonstrated that whiskey lactone has an enhancing effect on fruity notes at sub-threshold concentration and a making effect at supra-threshold concentration (Atanasova 2004). Thus, it could also have a masking effect on chlorine odour when delivered at just supra-threshold concentrations.

2. Material and Methods

2.1. Subjects and sensory procedure

A panel composed of 20 out of 48 subjects has been selected on the basis of screening tests including (i) the European test of olfactory capabilities (ETOC, Thomas-Danguin et al. 2003), (ii) a mental concentration test (Bourdon Test, Lesschaeve 1997) and (iii) their ability to rate various concentrations of chlorine odour and 1-butanol odour delivered using a computer-controlled air-dilution olfactometer (OM4b; Burghart instruments, Wedel, Germany). Then, panellists were trained to rate odour intensity on a 1-butanol reference scale which offers ratio properties (Atanasova et al. 2005a; Atanasova et al. 2004). Thus, panellists participated at 4 training sessions dedicated to the learning of 1-butanol references which were the scale
anchors. Afterwards, panel members were trained to rate various odour intensities of several compounds including hypochlorous acid.

During the 3 sessions dedicated to measurements (one session by compounds), subjects had to rate intensity, quality and liking for 3 concentration levels of hypochlorous acid, 5 concentration levels of the selected neutralising compounds and the 15 corresponding mixtures. All the measurements were performed according to the methodology presented in Atanasova (2004). Compounds and mixtures were delivered using a computer-controlled air-dilution olfactometer (OM4b; Burghart instruments, Wedel, Germany; Le Berre et al. 2008; Ishii et al. 2008).

Cinnamaldehyde (≥ 98%, Aldrich), 3-butylidenephtalide (≥96%, Aldrich) and whiskey lactone (≥98% Aldrich) were set pure in a dedicated olfactometer chamber. A Sodium Hypochlorite solution (120g/L Cl₂; NaOCl ~15%, RECTAPUR, VWR international, France) was introduced in a fourth chamber. To avoid solution depletion (Beauchamp et al. 2010), sodium hypochlorite solution was replaced before each session. Within the olfactometer, purified dry air went through the liquid to produce odorized air. This odorized air was diluted with humidified odourless air at the outlet of the olfactometer. Stimulus flow / total air flow ratio used are presented in Table 1. Physicochemical controls of odorant concentrations in the gas phase delivered by the olfactometer were conducted using GC-FID analyses and calibration curves (see details in Ishii et al., 2008).

<table>
<thead>
<tr>
<th>Concentration levels</th>
<th>Ratio (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL-1 Chlorine level 1</td>
<td>12%</td>
</tr>
<tr>
<td>CL-2 Chlorine level 2</td>
<td>34%</td>
</tr>
<tr>
<td>CL-3 Chlorine level 3</td>
<td>100%</td>
</tr>
<tr>
<td>Can-1 Cinnamaldehyde 1</td>
<td>0.4%</td>
</tr>
<tr>
<td>Can-2 Cinnamaldehyde 2</td>
<td>0.7%</td>
</tr>
<tr>
<td>Can-3 Cinnamaldehyde 3</td>
<td>1.5%</td>
</tr>
<tr>
<td>Can-4 Cinnamaldehyde 4</td>
<td>2.9%</td>
</tr>
<tr>
<td>Can-5 Cinnamaldehyde 5</td>
<td>5.8%</td>
</tr>
<tr>
<td>Cel-1 3-butylidenptalide</td>
<td>0.3%</td>
</tr>
<tr>
<td>Cel-2 3-butylidenptalide</td>
<td>0.6%</td>
</tr>
<tr>
<td>Cel-3 3-butylidenptalide</td>
<td>1.1%</td>
</tr>
<tr>
<td>Cel-4 3-butylidenptalide</td>
<td>2.2%</td>
</tr>
<tr>
<td>Cel-5 3-butylidenptalide</td>
<td>4.4%</td>
</tr>
<tr>
<td>WL-1 Whiskey lactone level 1</td>
<td>0.3%</td>
</tr>
<tr>
<td>WL-2 Whiskey lactone level 2</td>
<td>0.6%</td>
</tr>
<tr>
<td>WL-3 Whiskey lactone level 3</td>
<td>1.2%</td>
</tr>
<tr>
<td>WL-4 Whiskey lactone level 4</td>
<td>2.4%</td>
</tr>
<tr>
<td>WL-5 Whiskey lactone level 5</td>
<td>4.7%</td>
</tr>
</tbody>
</table>

Table 1: Dilution ratios used for the different odorants (Stimulus flow / total air flow)
2.2. Data analyses

The mean intensity ratings of the 20 subjects were calculated for each mixture, pure compounds and a blank (pure air). Additionally, as reported in previous studies on binary mixtures (Olsson 1994; Atanasova 2004; Patte and Laffort 1979) the composite variables $\sigma$ and $\tau$ were calculated for all mixtures.

Experimental data were thus reported on a graph based on these two parameters ($\sigma = f(\tau)$). $\tau$ reflects the relative proportion of perceived intensity of one unmixed odorant into the binary mixture. $\tau$ is the ratio between the perceived intensity of one of the odorants (e.g. odorant A) and the sum of the perceived intensities of the unmixed odorants: $\tau_A = I_A / (I_A + I_B)$. When $\tau = 0.5$, the mixture is iso-intense. $\sigma$ reflects the degree of overall intensity addition in the mixture. $\sigma$ is the ratio between the perceived intensity of one of the odorants (e.g. odorant A) and the sum of the perceived intensities of the unmixed odorants: $\sigma = I_{AB} / (I_A + I_B)$. Thus, when $\sigma = 1$, there is complete intensity addition, when $\sigma > 1$, there is hyper-addition and when $\sigma < 1$, there is hypo-addition. Representing $\sigma$ as a function of $\tau$ allows also to determine if there is subtraction, compromise or partial addition.

3. Results and Discussion

3.1. Physicochemical controls

Gas phase analyses were performed to measure odorant concentrations delivered at the outlet of the olfactometer through GC-FID analyses. Results are presented in Table 2. It is important to notice that these measurements evidenced a degradation of cinnamaldehyde which probably suffered from oxidation and formed benzaldehyde among other sub-products.
Table 6: Concentration in gas phase measured for the different stimulation levels

<table>
<thead>
<tr>
<th>Concentration levels</th>
<th>Ratio (v/v)</th>
<th>ppb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL-1 Chlorine level 1</td>
<td>12%</td>
<td>1</td>
</tr>
<tr>
<td>CL-2 Chlorine level 2</td>
<td>34%</td>
<td>11</td>
</tr>
<tr>
<td>CL-3 Chlorine level 3</td>
<td>100%</td>
<td>34</td>
</tr>
<tr>
<td>Can-1 Cinnamaldehyde 1</td>
<td>0.4%</td>
<td>71</td>
</tr>
<tr>
<td>Can-2 Cinnamaldehyde 2</td>
<td>0.7%</td>
<td>142</td>
</tr>
<tr>
<td>Can-3 Cinnamaldehyde 3</td>
<td>1.5%</td>
<td>285</td>
</tr>
<tr>
<td>Can-4 Cinnamaldehyde 4</td>
<td>2.9%</td>
<td>570</td>
</tr>
<tr>
<td>Can-5 Cinnamaldehyde 5</td>
<td>5.8%</td>
<td>1141</td>
</tr>
<tr>
<td>Cel-1 3-butylidenphtalide</td>
<td>0.3%</td>
<td>54</td>
</tr>
<tr>
<td>Cel-2 3-butylidenphtalide</td>
<td>0.6%</td>
<td>108</td>
</tr>
<tr>
<td>Cel-3 3-butylidenphtalide</td>
<td>1.1%</td>
<td>217</td>
</tr>
<tr>
<td>Cel-4 3-butylidenphtalide</td>
<td>2.2%</td>
<td>434</td>
</tr>
<tr>
<td>Cel-5 3-butylidenphtalide</td>
<td>4.4%</td>
<td>868</td>
</tr>
<tr>
<td>WL-1 Whiskey lactone level 1</td>
<td>0.3%</td>
<td>12</td>
</tr>
<tr>
<td>WL-2 Whiskey lactone level 2</td>
<td>0.6%</td>
<td>24</td>
</tr>
<tr>
<td>WL-3 Whiskey lactone level 3</td>
<td>1.2%</td>
<td>44</td>
</tr>
<tr>
<td>WL-4 Whiskey lactone level 4</td>
<td>2.4%</td>
<td>53</td>
</tr>
<tr>
<td>WL-5 Whiskey lactone level 5</td>
<td>4.7%</td>
<td>175</td>
</tr>
</tbody>
</table>

3.2. Sensory results

Only intensity data are reported. The mean intensity of each mixture, pure compounds and a blank (pure air) was reported on Figure 1, 2 and 3 respectively for cinnamaldehyde (degraded), 3-butylidenephtalide and whiskey lactone.

Figure 1: Mean intensity rating for the different levels of chlorine (CL), cinnamaldehyde (Can) and their mixtures.

Error bars represent 95% confidence interval. Red bars indicate mixture for which interaction has occurred.
Chapter III – Using perceptual odour interactions to reduce chlorine odour perception – Publication 9

Figure 2: Mean intensity rating for the different levels of chlorine (CL), 3-Butylideneephtalide (CEL) and their mixtures.

Error bars represent 95% confidence interval. Red bars indicate mixture for which interaction has occurred.

Figure 3: Mean intensity rating for the different levels of Chlorine (CL), Whiskeylactone (WL) and their mixtures.

Error bars represent 95% confidence interval. Red bars indicate mixture for which interaction has occurred.

These results showed that, despite preliminary adjustment studies, the intensity of chlorine odour was always very low whereas the intensity of putative neutralising compounds cover a quite large range of intensities. As a consequence, there was no actual difference between
chlorine odour intensity levels (CL_1, CL_2, CL_3) except in the case of celery odour (Butylideneptalide) as neutralising odour (Figure 2).

Results concerning perceptual interaction levels are presented in figure 4. On this figure, one can observe that interactions are evidenced in each mixture. Most often, intensity of odorant mixture followed the “U shape” (Patte and Laffort 1979). Thus, intensity of the mixture tended to follow the intensity of the main component. However, some points do not follow this shape. For example, compromise has been identified for mixture including whiskey lactone. Interactions observed with whiskey lactone are obtained for the highest concentration of hypochlorous acid (34 ppb) and the highest concentration of whiskey lactone (175 ppm).

These observations are consistent with the results reported by Atanasova (2004) who also observed masking of fruity notes when mixed with high whiskey lactone concentrations. Finally, we also evidenced that 3-butylideneptalide is likely to enhance chlorine odour. Thus, this strategy allowed us to identify compound likely to interact with chlorine. However, none of the observed interactions were evidenced at neutralising compound concentrations reconcilable with tap water distribution constraints. Indeed the intensity of the neutralising compound to use for neutralising properties to occur is far too high and unacceptable for tap water. Tap water must remain flavour-free as much as possible.
4. Conclusion

Despite issues in ratings performances and/or in stability neutralising compounds, our data evidenced perceptual odour interactions in studied binary mixtures. Thus, partial addition has been evidenced between chlorine and the salty associated 3-butylideneptalide odour. Compromises have been identified with cinnamaldehyde (degraded) and whiskey lactone odours. However, these interactions occur for high concentrations of neutralising compounds, not reconcilable with tap water consumption. Nevertheless, it appear that a selection of odorant based on specific associations encoded when odour have been experienced together could be a useful tool for screening of masking compounds.

Figure 4: \( \sigma = f (\tau) \) plots for mixtures of chlorine odour and a putative neutralising compound odour.

Each graph represents the binary interaction between chlorine and one of the compounds tested. Colours of the dots traduce intensity of the compound added to hypochlorous acid. Dot circled in red are the one for which interaction occurs.
4. Partial discussion

The aim of the experiments reported in this chapter was to screen aroma likely to reduce chlorine flavour perception. This was done in a first study in which a group of 30 tap water consumers and a group of bottled water consumers evaluated chlorine odour neutralising ability of 16 odorants initially selected for their differentiated functional groups. Result of this study did not allow to evidence any compounds likely to mitigate chlorine flavour perception or to increase chlorinated water liking or acceptability. In view of this failure, it appears that screening of masking compounds is an extremely difficult task and especially in the context of drinking water consumption because several criteria are required apart masking. Thus, we decided to conduct a more extensive study with compounds selected on the basis of their potential association with chlorine or compound sharing similar properties. Following such strategy, the 3 compounds used were found to interact with chlorine. Unfortunately, interactions evidenced did not occur in the required range of concentration. However, such strategy is still promising for selecting compound for screening purpose.

It is also important to remember that once again tap water consumers and bottled water consumers were found to differ in their chlorine flavour linking and acceptability. Thus, 70% of the tap water consumers would accept to daily drink the chlorinated solutions against only 48% of the bottled water consumers. Proportion of tap water consumers likely to accept chlorinated water decreased from 70% to 61% when aromas were added to water and this, whatever the aroma added. Such a noticeable decrease has to be taken into account for a future screening. Thus, acceptability of a potential neutralising compound could vary according to drinking water habits and representations.
General discussion and perspectives
General discussion and perspectives

This PhD has been conducted in the framework of the project “Eau bonne à boire” in partnership with the CIRSEE, the CESG and Robertet SA. This project aims to improve tap water organoleptic quality in order to better satisfy French consumers. One of the most important complaints reported about tap water relies on its unpleasant taste and especially its chlorine taste. Since chlorine is the only disinfectant to have residual properties for maintaining a good bacteriological quality from the treatment plant to the tap, it is not easy to replace it by another treatment. Several surveys on water quality published in the scientific literature pointed out organoleptic and safety reasons as factors explaining tap water dissatisfaction. Nevertheless, as highlighted by Doria (2006), they are very few studies based on blind tasting that were conducted to objectively measure water acceptability and sensitivity parameters. Therefore, the main question addressed during the PhD work was how to reduce chlorine flavour perception while maintaining an active chlorine concentration? To answer this question, our strategy was first to determine the sensory mechanisms underlying chlorine flavour perception and then to explore perceptual levers likely to reduce chlorine flavour perception. This exploration was conducted through two approaches. The first one consisted to determine whether sensory interactions could exist between chlorine flavour and water taste induced by the mineral matrix and to find the water composition parameters allowing minimal chlorine flavour perception. The second approach aimed to identify odorants that would be able to neutralize chlorine flavour at a perceptual level or to increase chlorinated water liking and/or acceptability.

The first part of the PhD work was dedicated to identify sensory modalities involved in chlorine flavour perception and to explore the link between chlorine flavour perception and water consumption habits. We set out to determine the sensory modalities involved in chlorine flavour perception by detection threshold measurements. Suprathreshold measurements were also performed to assess intensity, liking and acceptability for solutions including various chlorine concentrations. Our results demonstrated that chlorine flavour perception rely on activation of the olfactory system at low concentrations (>0.14 mg/L Cl₂) and activation of the trigeminal system at higher concentrations (>4.1 mg/L Cl₂). At concentrations lower than 3 mg/L Cl₂, the gustatory system was not found to be activated. Our results also evidenced that tap water consumers and bottled water consumers do not differ in their sensitivity to chlorine but differs in their liking of and their acceptability to drink chlorinated water. This suggests that chlorine flavour representations of tap water are determinant for consumer behaviour. Several studies even demonstrated that organoleptic properties of water shape safety perception (Slovic 1987, Jardine, Gibson et al. 1999). Acceptability of chlorinated solutions decreases as chlorine concentration increases. Moreover, acceptability differences between both consumers’ groups especially increase for concentrations likely to activate trigeminal nerve (>3 mg/L Cl₂). And, even if sensitivity
differences between both consumer groups have not been evidenced as far as trigeminal sensitivity is concerned, our data suggest that such sensitivity differences could exist depending on the stimulation route (i.e. retronasal vs. orthonasal). However, this hypothesis remains to be tested in further dedicated studies.

At this point, it is important to mention that Doria et al. (2005) demonstrated that risk perception associated with tap water consumption is explained by external information, such as colour, flavour, and past experience (memorability and familiarity). Syme & Williams (1993) found a weak but significant correlation between prior negative experiences and the acceptability of drinking water quality or risk. These authors especially evidenced that frequent water quality changes are inversely associated with water quality acceptability and water risk judgments leading to a decrease in consumer confidence. However, maintaining chlorine constant delivery in water networks is quite a difficult task. This is mainly due to change in water demands during the day. For example, in the Dijon area, while the target chlorine concentration at tap is 0.1 mg/L Cl₂, 10% of the water chlorine content measurements performed on the network had a concentration comprise between 0.3 and 1 mg/L Cl₂ (Data not shown). Indeed, everyone drinking tap water has already perceived changes in chlorine intensity at tap. Such kind of variations may have an impact on the perceived quality of water since they are not in favor of habituation mechanisms. Additionally, as reported by Slovic (2000), people who are familiarised with hazardous substances or activities perceive them to be less risky. However, water chlorination appears as being underestimated and not controllable. Therefore, it seems that maintaining chlorine level as most constant as possible could help to improve consumer acceptability.

The second chapter was dedicated to the study of cross-modal interactions likely to affect chlorine flavour perception with the aim to identify putative perceptual neutralization levers. If the determinants underlying chlorine detection were deciphered thanks to the first series of studies, determinants underlying drinking water taste remained unclear. Thus, several studies have been conducted (i) to determine the range of variations of the different water mineral composition factors and (ii) to determine their impact on drinking water taste perception. Thus, our studies evidenced that bottled water and tap water composition factors do not vary in the same extent: the range of molarity is more expanded in bottled waters than in tap waters. In the same way, a higher number of ionic pattern typologies have been identified in bottled waters than in tap waters. Our results showed cations are well discriminated despite the very low ionic content of drinking water (i.e. tap waters). Our result also confirmed the effect of the global mineral content of water (i.e. molarity or TDS) which has already been reported by several authors (Bruvold et al. 1967; Burlingame et al. 2007; Teillet et al. 2009a,b) demonstrated using sorting procedure that waters with a low mineral
content and high mineral content elicit tastes whereas water with a medium mineral content (≈ 10 mM; ≈ 300 mg/L) are perceived as neutral. These results were confirmed by our findings. We evidenced that waters containing a high proportion of sodium elicit a higher taste as compared to water with a high proportion of calcium.

Peripheral mechanisms could account for these findings. Indeed, Matsuo and Yamamoto (1992) demonstrated similar responses obtained for prototypical tastants solutions when the tongue is adapted to water or dialyzed saliva. On the opposite these responses differ from the responses obtained under saliva-adapted conditions. In further investigations, the authors evidenced that responses obtained under adaptation to the major ions contained in saliva (i.e. Na⁺, K⁺, Cl⁻, et HCO₃⁻) were similar to those obtained under saliva-adapted conditions. This was showed to be especially true after adaptation to NaHCO₃ – 10 mM. On the opposite, responses obtained after adaptation to the minor compounds of water (<5 meq/L; Ca²⁺, Mg²⁺, F⁻, I⁻, H₂PO₄⁻) did not differ from those obtained after adaptation to water. However, our results evidenced that Na⁺ is perceived at these concentrations. In such a context it is difficult to determine to what extent this mechanism could account for drinking water taste responses.

It is interesting to notice that water properties associated with perceived neutrality (≈10 mM and high proportion of Ca²⁺) are also waters the most frequently consumed. Indeed, results presented in publication 4 indicates that mean molarity of tap water delivered in France is 8.1 mM and that 82 % of the waters have an ionic pattern characterized by a high calcium proportion. For example, the Dijon area is supplied with water corresponding to the most common pattern namely a high proportion of Ca²⁺ and HCO₃⁻ and with a medium molarity level. This means that consumers included in the tap water groups and who participated to the tests presented in this PhD are supplied and therefore drink water which present the composition characteristics most often encountered in France. Taking into account this information, it is interesting to consider the results of an additional study (not reported in this manuscript as soon as it was performed out of PhD framework but within the whole research project) which examine water consumers liking and acceptability, for waters varying on mineral matrix composition. Indeed, these results indicated that water with low molarity and/or high sodium proportions are depreciated thus suggesting that drinking water perception and acceptability could rely on habituation mechanism (Thompson et al. 2001) which is a central mechanism. Teillet (2009) tested this hypothesis by measuring liking for water in 3 different areas supplied by waters with 3 different molarity levels and found a low but significant effect of the water consumption habits on liking. When taken together, all these results constitute a body of arguments to conclude that consumers supplied with a specific water composition are much inclining to appreciate this water composition.
General discussion and perspectives

When considering the interactions between water taste induced by its mineral content, our results clearly evidenced that chlorine flavour varied according to water mineral composition. These interactions seem to be due to physicochemical interactions. Indeed, we found that at a low molarity level, pH is the main factor modulating chlorine flavour. This modulation was explained by the influence of water pH on the quantity of hypochlorous acid associated form present in the headspace. At higher molarity levels, the results we obtained were far much difficult to explain. Nevertheless, it appeared that different type of physico-chemical interactions are probably involved, some due to the mineral matrix itself, some relying on in-mouth interactions probably with saliva. Saliva content in HCO$_3^-$ could modify the pH of the water samples once in the mouth and therefore affect chlorine flavour intensity. To confirm this hypothesis; further experiments are needed to measure HOCl content in headspace above different chlorinated water samples but also in the mouth-space. However, the methodology we used for chlorine headspace measurement (INRS, 2006) is not sensitive and specific enough to envisage such a study. Methodological developments are needed to ensure a correct level of sensitivity of physico-chemical analyses. One possibility would be to use *in vivo* APCI-MS measurements. Indeed, Foster *et al.* (1999) used APCI-MS to quantify HOCl in atmosphere.

Beyond physico-chemical interactions, perceptual interactions may also account for a part of our results. For example, chlorine flavour appears to be more intense in chlorinated waters at low and high molarity levels. In contrast, chlorine flavour intensity seems to be lower in chlorinated water at medium molarity level. Therefore, it appears that chlorine flavour may be dependant on water taste which was found to be neutral at medium molarity level. Nevertheless, we failed to evidence any significant relationship between chlorine flavour and water taste. This could be explained both by the cross-influence of physico-chemical interactions and by a high inter-individual variability induced by saliva effects. Additionally, it is interesting to consider the liking of chlorinated solutions. This has been done in the already mentioned side-study (not reported in the PhD). The results showed that liking scores for chlorinated samples decreased for water samples with a low molarity but also for water with a high proportion of sodium. Water taste / chlorine flavour interactions can account for these results, but consumption habits can also have an impact, since the most appreciated samples were close to water daily delivered at tap in the Dijon area. This hypothesis is supported by the results reported by Syme & Williams (1993) who evidenced that frequent changes quality are inversely associated with quality acceptability and water risk judgments. Once again and from an industrial point of view, the constancy in quality of water delivered at tap seems to be crucial.

As far as industrial point of view is concerned, it is important to underline that tap water is a multipurpose product which has to correspond to the needs of several uses, some being not...
easily reconcilable. Another complaint about tap water is its hardness that is its high content in calcium bicarbonates which is responsible for calcium carbonate scaling. The water softening treatments could be used. They consist in the reduction of calcium bicarbonates concentration that is a decrease in the concentration of Ca\(^{2+}\) and HCO\(_3^-\) ions. However, our data demonstrated the importance of these ions in chlorine flavour perception. Therefore, water softening would conduct to water with a lower molarity which would be less accepted because of its taste and/or for its more intense chlorine flavour.

Beside the putative influence of water mineral content on chlorine flavour perception, we explored the possibility to use odours to mitigate chlorine flavour. Our results evidenced perceptual interactions between chlorine flavour and putative neutralizing compound odour. However none of the tested compounds did reduced significantly chlorine flavour or increased chlorinated water liking or acceptability. In view of this failure, it appears that screening of masking compounds is an extremely difficult task and especially in the context of drinking water consumption because several constraints have to be considered. Despite several experimental problems, our last study suggested a way to screen potential neutralizing compounds. Indeed, in this study, we assessed the neutralizing ability of 3 odorants selected on the basis of their potential perceptual interaction with chlorine or compounds sharing similar perceptual properties. The very preliminary findings suggested that this strategy should be most relevant for such king of issues and could be useful for future screenings. However, it is important to remember that our results showed that aroma addition in tap water would have a negative impact on tap water consumer satisfaction. Indeed, the proportion of tap water consumers likely to accept aromatized chlorinated water decreased from 70% to 61%. This also pleads in favour of the importance of habituation as far tap water is concerned. It is also important to notice that surveys conducted to understand determinants of drinking water evidenced that tap water appears as a technical product highly processed. On the opposite, bottled water appears, thank to marketing, as a more natural product which pleads in its favour. Thus, adding aroma to tap water could constitute a limitation to tap water consumption. Acceptability of the concept of aroma addition has to be studied in deep.

To conclude, neutralizing chlorine flavour in tap water remains a scientific and industrial challenge. The knowledge obtained within the framework of this PhD may contribute to find solutions. The perceptual mechanisms implied in chlorine flavour perception are elucidated. This should help to identify putative neutralizing levers. Indeed, we found that chlorine flavour did not rely on taste but that cross-modal interaction between water taste and chlorine flavour could be observed. If an ideal water mineral matrix composition recipe could not be found, several interesting findings may help to adapt mineral composition to better satisfy
General discussion and perspectives

consumers expectations in terms of water organoleptic qualities or other functionalities. The neutralizing lever relying on aroma addition is an interesting way but needs several complementary investigations to reveal its actual efficiency and acceptability for the consumers. Finally, one main aspect to be taken into account and which has weakly been explored within this PhD work is the importance of habituation and constancy on consumer acceptability. Additionally, an increase of tap water acceptability as drinking water may goes through an improvement of tap water representations.
A


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C


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